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METAL IONS IN MONOAMINE FLUORESCENCE HISTOCHEMISTRY

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The present dissertation is based on the following papers:

1. LÖRÉN, I., A. BJÖRKLUND, B. FALCK and O. LINDVALL: An improved histofluorescence procedure for freeze-dried paraffin-embedded tissue based on combined formaldehyde-glyoxylic acid perfusion with high magnesium content and acid pH. Histochemistry 49, 177-192, 1976.
2. LÖRÉN, I., A. BJÖRKLUND and O. LINDVALL: Magnesium ions in catecholamine fluorescence histochemistry. Application to the cryostat and Vibratome techniques. Histochemistry 52, 223-239, 1977.
3. LÖRÉN, I., A. BJÖRKLUND, B. FALCK and O. LINDVALL: The aluminum-formaldehyde (ALFA) histofluorescence method for improved visualization of catecholamines and indoleamines. 1. A detailed account of the methodology for central nervous tissue using paraffin, cryostat or Vibratome sections. J. Neurosci. Meth. 2, 277-300, 1980.
4. AJELIS, V., A. BJÖRKLUND, B. FALCK, O. LINDVALL, I. LÖRÉN and B. WALLES: Application of the aluminum-formaldehyde (ALFA) histofluorescence method for demonstration of peripheral stores of catecholamines and indolamines in freeze-dried paraffin-embedded tissue, cryostat sections and whole-mounts. Histochemistry 65, 1-15, 1979.
5. LÖRÉN, I., A. BJÖRKLUND, O. LINDVALL and R.H. SCHMIDT: Improved catecholamine histofluorescence in the developing brain based on the magnesium and aluminum (ALFA) perfusion techniques: methodology and anatomical observations. Accepted for publication in Brain Research Bulletin.
6. BJÖRKLUND, A., B. FALCK, O. LINDVALL and I. LÖRÉN: The aluminum-formaldehyde (ALFA) histofluorescence method for improved visualization of catecholamines and indoleamines. 2. Model experiments. J. Neurosci. Meth. 2, 301-318, 1980.
7. LINDVALL, O., A. BJÖRKLUND, B. FALCK, I. LÖRÉN and L.-Å. SVENSSON: New fluorophore-forming reactions for histochemical visualization of N-acetylated and tertiary indolamines using glyoxylic acid, aluminum-formaldehyde and trifluoroacetic acid anhydride as reagents. Accepted for publication in Histochemistry.

INTRODUCTION

Several fluorescence histochemical techniques are today used for the demonstration of the catecholamines (CA:s), noradrenaline (NA) and dopamine (DA), and the indolamine, 5-hydroxytryptamine (5-HT), in neuronal and non-neuronal tissue. The fluorescence histochemical techniques currently in use are all based on the formation of fluorophores in reactions between the reagents formaldehyde (FA; chemical formula HCHO) and/or glyoxylic acid (GA; chemical formula HOOCCHO) and respective amine.

The first report of a specific fluorescence in certain cells - the enterochromaffin cells, mast cells and carcinoid cells described as "die gelbe Zellen" - after formalin fixation was published by Erös (1932). However, due to the limited knowledge of biogenic monoamines, these observations remained obscure. Over 20 years later the fluorogenic compound in these cells was identified as 5-HT (Barter and Pearse 1953, 1955), which in the reaction with FA forms a compound that exhibits a yellow fluorescence in the microscope upon illumination with ultraviolet light.

After fixation in liquid formalin certain cells of the adrenal medulla exhibited a green fluorescence when examined in a fluorescence microscope (Eränkö 1952). These cells were later shown to be noradrenaline-storing (Eränkö 1955 a-c). The findings made by Eränkö opened up an entirely new and important field within histochemistry. Erös and Eränkö were the first to describe FA induced fluorescence in 5-HT- and NA-storing cells. However, the sensitivity of their techniques were far from that level needed for demonstration of intraneuronal biogenic monoamines. The real breakthrough in monoamine fluorescence histochemistry came in the early sixties when Falck, Hillarp and co-workers (see below) applied gaseous FA to dried tissue specimens and succeeded in visualizing catecholamines and 5-HT at their intraneuronal storage sites.

Freeze-drying and paraffin embedding procedures

The fluorescence histochemical method for freeze-dried, paraffin-embedded tissue, i.e. the Falck-Hillarp method, which was developed by Falck, Hillarp and co-workers (Falck and Torp 1961, Falck 1962, Falck et al. 1962; described in detail by Falck and Owman 1965, Eränkö 1967.

Fuxe et al. 1970, Jonsson 1971, Björklund et al. 1972a), represents a highly sensitive methodological approach for demonstration of NA, DA and 5-HT in tissue. The method is based on freeze-drying, condensation with gaseous FA, paraffin embedding and sectioning.

Methodological limitations within the original Falck-Hillarp method have made improvements of this procedure a necessity. The combined FA-ozone-reaction (Björklund et al. 1968b, Björklund and Falck 1969) demonstrates tryptamine and peptides with NH_2 -terminal tryptophan (Håkanson et al. 1972, 1974 and 1975) with an increased sensitivity. In the acid-catalyzed FA reaction the sensitivity is enhanced in the visualization of peptides with NH_2 -terminal tryptophan as well as of tryptophan, tryptamine and certain tryptamine derivatives, and of 3-methoxylated β -phenylethylamines (Björklund et al. 1968b, Björklund and Falck 1969, Björklund and Stenevi 1970, Björklund et al. 1971, Partanen et al. 1974) FA condensation at reduced temperature offers an improved sensitivity for demonstration of NA and DA and improved discrimination between the primary catecholamines (NA and DA) and adrenaline and between primary catecholamines (NA and DA) and tryptamine or peptides with NH_2 -terminal tryptophan (Håkanson and Sundler 1974).

To obtain a more reproducible fluorescence picture standardization of the humidity of the paraformaldehyde powder, from which the FA vapour should be generated, proved to be of importance (Hamberger 1967). A further improvement of the visualization of 5-HT in the central nervous system was achieved by a modified FA vapour treatment (Fuxe and Jonsson 1967), which is based upon a prolonged exposure to a more humid FA vapour than used for NA and DA visualization. Certain delicate DA-containing nerve fibers are more sensitive for several steps in the routine histological processing. By shortening the time for vacuum embedding and modifying the deparaffinizing and mounting steps, the reproducibility in the Falck-Hillarp method for certain delicate DA-fiber systems was improved (Björklund and Falck 1968).

Intravascular perfusion with a chilled hypertonic salt solution prior to dissection and further processing of tissues proved to be another way of increasing the sensitivity in the Falck-Hillarp method. Such a perfusion procedure increases the fluorescence intensity, e.g., in cortical NA and DA systems (for further comments see

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Hökfelt and Ljungdahl 1972) and was used for the demonstration of terminal systems in hippocampus (Blackstad et al. 1967), in cerebral cortex (Fuxe et al. 1968) and cerebellum (Hökfelt and Fuxe 1969).

GA is a more sensitive reagent than FA for visualization of NA and DA in tissue (Lindvall and Björklund 1974) but has a limited penetration capacity for freeze-dried tissue blocks. Thus, GA vapour treatment cannot be used in the same way as FA vapour for developing the NA and DA fluorophores in such specimens. However, if the specimens are freeze-dried, vacuum embedded, paraffin sectioned and gently deparaffinized before exposure to GA vapour treatment, endocrine cell systems storing peptides with NH_2 -terminal tryptophan (Björklund et al. 1973b), sympathetic nerves, 5-HT containing mast cells and enterochromaffin cells are shown with a high sensitivity (Axelsson et al. 1973). The demonstration of catecholamine nerve fibers in the central nervous system using this GA procedure did not show any advantages compared to the original Falck-Hillarp method.

Cryostat procedures

The cryostat has proven to be very useful in fluorescence histochemistry. This instrument is accessible to most laboratories. Furthermore, the methods based on cryostat sectioning are easy to perform, do not require special equipments (as needed in the freeze-drying techniques) and allow easy serial sectioning together with parallel processing of several tissue specimens. Although many efforts have been made to develop a highly sensitive cryostat technique for the histochemical demonstration of NA, DA and 5-HT, application to cryostat sections still offers the least sensitive variant of all methods used for this purpose. Typical drawbacks are inconsistent reproducibility, diffusion artefacts and difficulties in obtaining good section morphology at the low temperatures needed to obtain a good fluorescence picture. Cryostat sections have been used in several published procedures in order to make use of the practical advantages of the cryostat instrument. The published cryostat methods are all based upon fluorophore formation by use of the reagents FA or GA, or a combination of both.

The formation of FA induced fluorescence from CAs in cryostat sections was first described by Eränkö (1955 a-c). Eränkö reported that immersion of adrenal glands in a formalin solution followed by cryostat sectioning demonstrated NA-containing

cells in the adrenal medulla. It is notable, that no FA vapour treatment was needed for the formation of the NA fluorophore (see above and in discussion). Similar results have been obtained in cardiac tissue after intracardial perfusion with a 4% paraformaldehyde solution just prior to cryostat sectioning (Laties et al. 1967). These authors also reported that heating of the sections further improved the fluorescence picture. However, the best results were obtained after FA vapour treatment according to the Falck-Hillarp method.

During the sixties and early seventies several attempts were made to apply the Falck-Hillarp FA vapour method to cryostat sections. Most of them were based upon rapid freezing of freshly dissected tissue specimens, sectioning in the cryostat microtome, drying of the sections and finally development of the fluorophores by FA vapour treatment (Hamberger and Norberg 1964, Spriggs et al. 1966, Csillik and Kálmán 1967, El-Badawi and Schenk 1967, Heene 1968, Nelson and Wakefield 1968, Placidi and Masuoka 1968, Winckler 1970). Most of these authors use a modified freeze-drying of the freshly cut sections in order to enhance the fluorescence picture and to reduce the diffusion of fluorophores.

A further increased sensitivity in the FA-cryostat methods was obtained after intracardial perfusion of the animals with a 4% paraformaldehyde solution just prior to dissection (Laties et al. 1967, Watson and Ellison 1976). Perfusion of the tissue proved to reduce the problems of diffusion and to favour more reproducible results. This was demonstrated in NA-containing nerves of cardiac tissue (Laties et al. 1967) and in NA-, DA- and 5-HT-containing neurons of the central nervous system (Watson and Ellison 1976). The failure to visualize neurons in the central nervous system by Laties et al. depended probably on the rather high sectioning temperature (-10°C) as compared to that used by Watson and Ellison (1976) (-25°C).

The novel reagent, GA, has proven very efficient in the demonstration of peripheral and central neuronal storages of NA, DA and 5-HT in cryostat sections obtained from non-perfused tissue (de la Torre and Surgeon 1976a, b, Watson and Barchas 1977, de la Torre 1980). These methods are based on the development of the fluorophores in either of the two ways as described for the original GA Vibratome method (Lindvall and Björklund 1974; see below). While de la Torre recommends heating of GA immersed sections, Watson and Barchas use GA vapour treatment. Both methods allow for rapid processing of sections for fluorescence microscopy.

This is especially notable in de la Torre's SPG-method, where sections can be obtained for fluorescence microscopy after 18 minutes.

Perfusion of the tissue with a cold GA-containing buffer proved to enhance the sensitivity of the cryostat methods for NA and DA in the central nervous system (Battenberg and Bloom 1975, Bloom and Battenberg 1976). After perfusion via the ascending aorta with a 2% GA and 0.5% FA solution, tissue samples were dissected, frozen in dry ice and sectioned in the cryostat. The sections were then immersed in a 2% GA solution, dried and the fluorophores developed by heating. This method was further standardized and improved (less diffusion, higher reproducibility and sensitivity) by reducing the time of GA immersion of the sections and by exposure of the immersed sections to FA vapour for 1 hour according to the Falck-Hillarp method (Nygren 1976).

Vibratome procedures

Hökfelt and Ljungdahl (1972) introduced the Vibratome instrument for monoamine fluorescence histochemistry, and they described a modification of the Falck-Hillarp method using FA perfused tissue. This FA-Vibratome method had a markedly enhanced sensitivity for visualization of NA and DA containing neurons in central nervous tissue. The method is based on perfusion with a 4% ice-cold formalin solution, sectioning on the Vibratome instrument, drying, and FA vapour treatment. Besides its enhanced sensitivity this procedure is also much less time consuming than the original Falck-Hillarp method by the elimination of the freeze-drying step. The replacement of FA with GA in the Vibratome technique (Lindvall et al. 1973, Lindvall and Björklund 1974) represents a further increased sensitivity in histofluorescence demonstration of NA-and especially DA-containing neuron systems in the brain (Lindvall and Björklund 1974, Lindvall and Björklund 1978). The GA-Vibratome method is based on intravascular perfusion with an ice-cold 2% GA solution, sectioning on the Vibratome instrument, immersion in a 2% GA solution, drying and development on the fluorophores either by heating in an oven, or by exposure to GA vapour. By combining the GA-Vibratome method (omitting of the heating step or GA vapour treatment) with exposure of the dried sections with FA vapour, Berger et al. (1976) obtained a further improved visualization of cortical NA-containing nerve fibers.

Recently, an interesting and very promising development of the aldehyde fluore-

science method, has been achieved – particularly through modifications of the fixation procedures – for combined fluorescence- and electron microscopy of CA-containing structures. Chiba and Williams (1975; Chiba et al. 1976) worked out a combined GA-FA-glutaraldehyde procedure for combined studies on 5HT-cells in sympathetic ganglia. In this method the specimens are either perfused or immersed in two steps, first with a 2% GA solution and then with an aldehyde mixture consisting of 0.5% glutaraldehyde and 4% FA. After cutting on the Vibratome instrument the sections are then transferred for combined fluorescence and electron microscopy examination by omitting of the drying and heating steps, which are performed if the sections only are taken for fluorescence microscopy. Furness et al. (1977a, 1978) introduced a new aldehyde mixture, consisting of FA and glutaraldehyde (the Faglu mixture), using cryostat or Vibratome sectioning. The brains are fixed by perfusion with the solution (1/2% glutaraldehyde and 4% FA), sectioned and processed for fluorescence and electron microscopy either on the same or the consecutive section. In this method the fluorophores are developed in the Faglu solution. Glutaraldehyde is believed to bind to, and thereby stabilize, the fluorophore in the aqueous phase, which is of great importance, since inadequate drying of the section breaks this bond and impairs the result. A thorough drying is thus needed for those sections which are used only for fluorescence microscopy.

Procedures based on whole-mount preparations

For the demonstration of NA and 5-HT in their neuronal as well as non-neuronal storage sites, so-called whole-mount preparations are of great advantage for certain kinds of tissue. This preparative technique is simple and rapid. Whole-mounts are often used in combination with sectioned specimens processed according to other variants of the aldehyde histofluorescence methodology in order to obtain a complete neuroanatomical picture of the innervation of certain organs. Suitable organs are those which are possible to spread (either directly or after separation into subcompartments) as thin sheets (e.g., iris, meninges, capsules, blood vessels, the gastrointestinal tract etc). The original procedure for the development of NA and 5-HT fluorescence, devised by Falck in 1962, involved drying of the freshly dissected tissue in vacuo over P_2O_5 followed by exposure to FA vapour. This procedure has subsequently been used in a number of elegant neuroanatomical studies

of the peripheral sympathetic system, e.g., Malmfors (1965), Nielsen and Owman (1967), Olson (1967, 1969), Furness and Costa (for a detailed reference list see Furness and Costa 1974) and Lindvall and Owman (1978). Whole-mounts have also shown suitable to be processed according to the standard Falck-Hillarp method, i.e. the specimens are dissected, freeze-dried and FA vapour treated (Mc Lean and Burnstock 1966, Read and Burnstock 1968).

A similar principle forms the basis for using tissue "smears" of punched brain regions, since such tissue cannot be used for whole-mount preparations (Olson and Ungerstedt 1970). This method demonstrates fluorescent varicosities containing NA, DA and 5-HT but not nerve cell bodies or nerve fibers. The visualization of CA varicosities is improved if the brains are perfused with an ice-cold 4% formalin solution before dissection and punching (Lorén, unpublished observations). After smearing of the tissue, the slides are dried and reacted with FA vapour.

Both the GA and Faglu methods have subsequently been applied with excellent results on whole-mount preparations. GA has proven to be a preferable reagent in comparison with FA for the fluorescence histochemical demonstration of CAs and 5-HT in whole-mounts. Thus, when such tissue samples were immersed in a 2% GA solution (0-18°C; pH 7), dried and exposed to heat or GA vapour, a clear cut improvement of the fluorescence picture was obtained (Furness and Costa 1975, Lindvall and Björklund 1974, Waris and Partanen 1975). The investigations carried out by Furness and Costa (1975), Waris and Partanen (1975), Stefenson et al. (1981) showed an enhanced sensitivity in the demonstration of NA in the peripheral nervous system. Furthermore, acidification of the GA immersion solution (pH 3.5) enhanced the sensitivity and fluorescence intensity of 5-HT in its peripheral storage sites compared to that obtained in a neutral GA immersion solution (pH 7) (for further details see Furness and Costa 1975).

The fluorophore forming capacity of an aqueous medium containing FA (4%) and glutaraldehyde (0.5%), i.e. the Faglu mixture, can be used for sensitive demonstration of NA, DA and 5-HT in the fluorescence microscope concomitant with electron microscopy of the same tissue sample (Furness et al. 1977b). In this fluorescence method no FA vapour treatment is needed for the development of the monoamine fluorophore. The fluorophores are formed in the room-tempered Faglu mixture during 1-3 hours of immersion. The fluorescence picture obtained is

improved in comparison with the previously described FA methods for whole-mounts (see above). If the tissue is dried over P_2O_5 the fluorescence picture is further improved to that level, which only has been possible to gain with the GA methods applied to whole-mounts (see above).

Immunohistochemical methods

In parallel with the methodological improvements within aldehyde fluorescence histochemistry, immunohistochemical procedures have been developed for the demonstration of CA and indolamine neurons. The immunocytochemical methods for CA neurons are based upon the use of antibodies raised against the various enzymes in CA synthesis. Several reports describing the distribution of tyrosine hydroxylase (TH) (Hökfelt et al. 1975, 1976, 1977, Pickel et al. 1976), dopa decarboxylase (DDC) (Hökfelt et al. 1973a, b, 1975), dopamine- β -hydroxylase (DBH) (Fuxe et al. 1971, Hartman 1973, Hartman et al. 1972, Swanson and Hartman 1975, Hökfelt et al. 1973 b, 1975) and phenylethanolamine-N-methyl transferase (PNMT) (Fuxe et al. 1971, Hökfelt et al. 1973 b, 1975) have been made. The immunohistochemical demonstrations of the CA synthetic enzymes are very sensitive and visualizes the CA neuronal systems with a richness of details and a sensitivity not far from that obtained with the most sensitive aldehyde methods. Recently, antibodies have been raised against serotonin (5-HT) (Steinbusch et al. 1978) and melatonin (Bubenik et al. 1974, 1976). The antibodies raised against serotonin reveals the distribution pattern in the CNS of 5-HT with a much higher precision than has been the case with any of the aldehyde methods (Steinbusch 1981, Hökfelt et al. 1978).

Chemical background of the fluorophore-forming reactions

The reaction-mechanisms and reaction products in the Falck-Hillarp and in the GA histofluorescence methods have been thoroughly investigated (see Corrodi and Jonsson 1967, Jonsson 1967, Björklund et al. 1972b, 1975). The fluorophore formations proceeds in two steps. In the first step, which is a Pictet-Spengler cyclization reaction the ring closure of the amine is facilitated by high electron density at the 2-position of indolylamines and at the 6-position of phenylethylamines (Fig. 1). This is the case for 3-hydroxylated β -phenylethylamines and for β -(3-indolyl)-ethylamines.

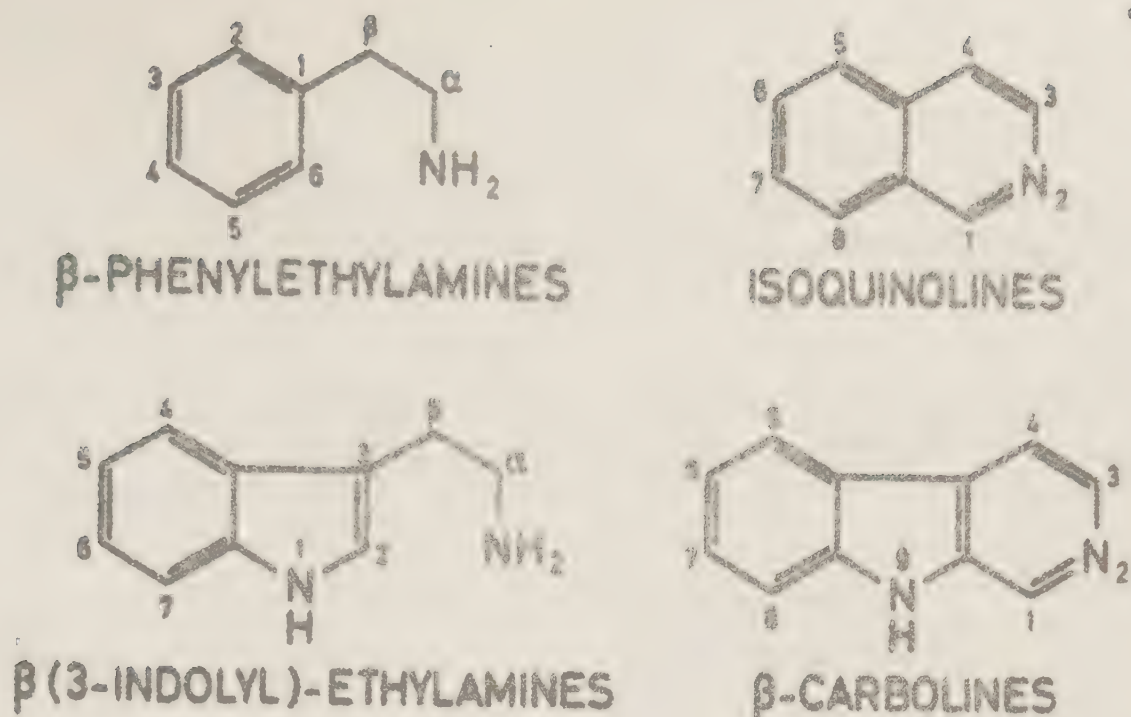


Figure 1. Labelling and positions in phenylethylamine and indolyethylamine derivatives and in their corresponding isoquinoline and β -carboline. (Reproduced from Björklund et al. 1975).

Unsubstituted indolyethylamines are less reactive than those having a hydroxy or methoxy group in the 5-position of the indole nucleus. The Pictet-Spengler reaction is restricted to primary and secondary amines. The Pictet-Spengler condensation reaction between FA and primary catecholamines, such as DA and NA (Fig. 2a), occurs very rapidly resulting in the formation of the very weakly fluorescent tetrahydroisoquinoline derivatives (I and IV in Fig. 2a). In a second step the tetrahydroisoquinolines are dehydrogenated to their corresponding dihydroisoquinolines (II and V in Fig. 2a; $\text{R}=\text{H}$). This second reaction step proceeds either via an autoxidation, or via an acid catalyzed step with another FA molecule to yield a 2-methyl-dihydroisoquinoline (II and V in Fig. 2a; $\text{R}=\text{CH}_3$) (Björklund et al. 1973a). The dihydroisoquinolines can exist in a non-quinoidal form (acid milieu; II and V in Fig. 2a) or in a quinoidal form (neutral or alkaline milieu; III and VI in Fig. 2a). The 4-hydroxy group on the NA-fluorophore can be splitted off with acid and the NA-fluorophore is then transferred into the fully aromatic compound 6,7-dihydroisoquinoline (VII in Fig. 2a), which is followed

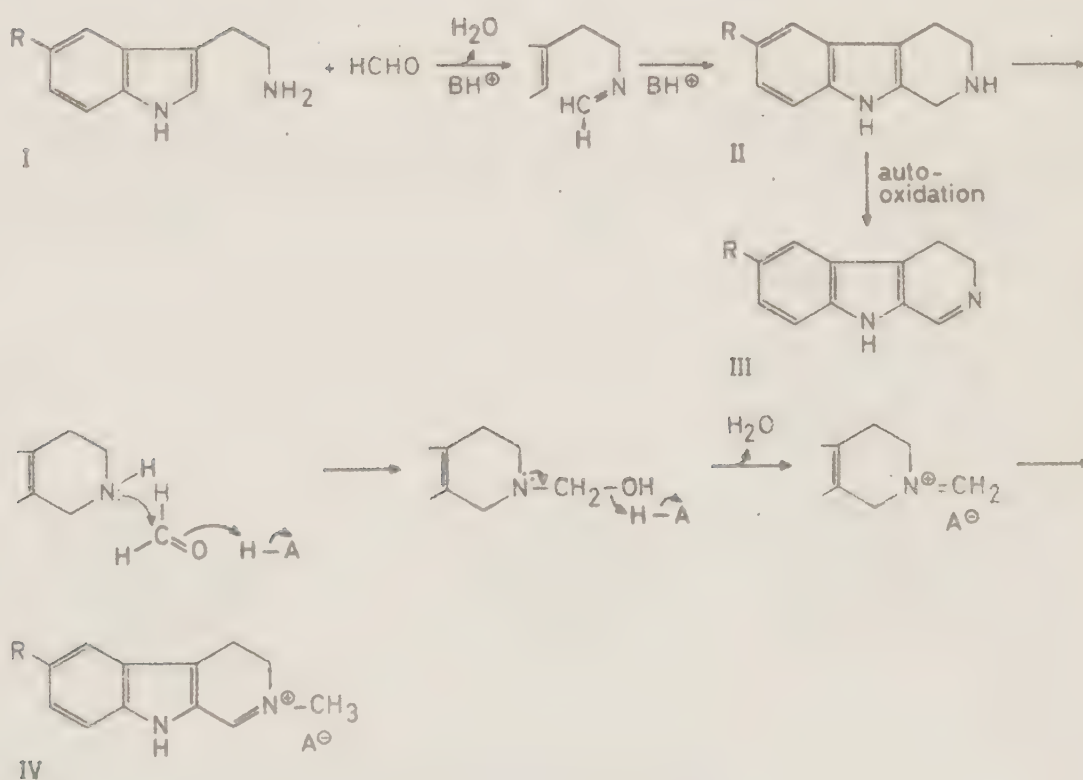
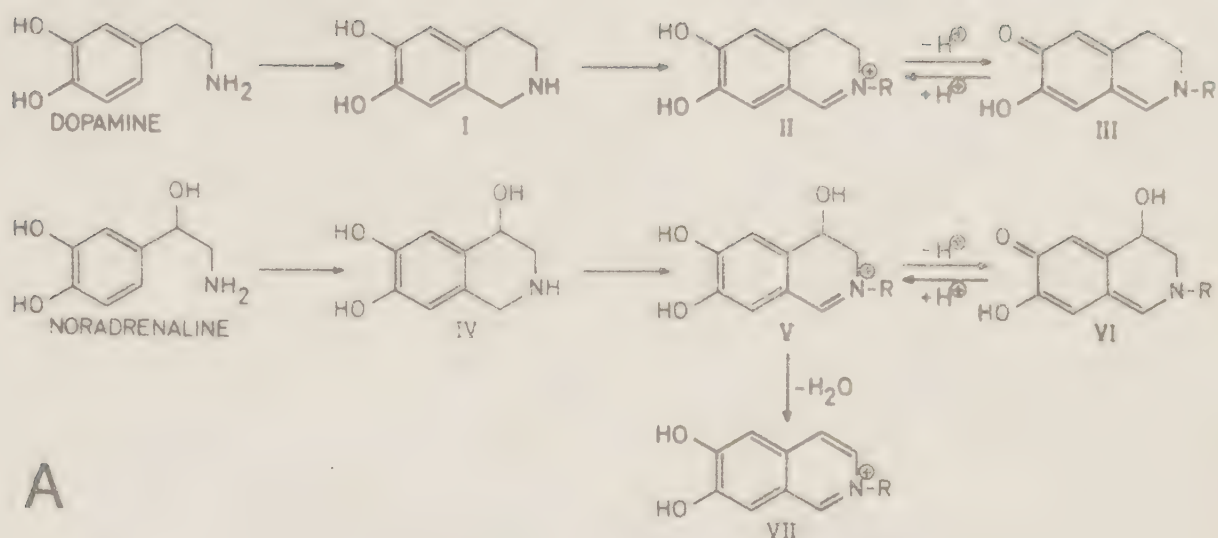
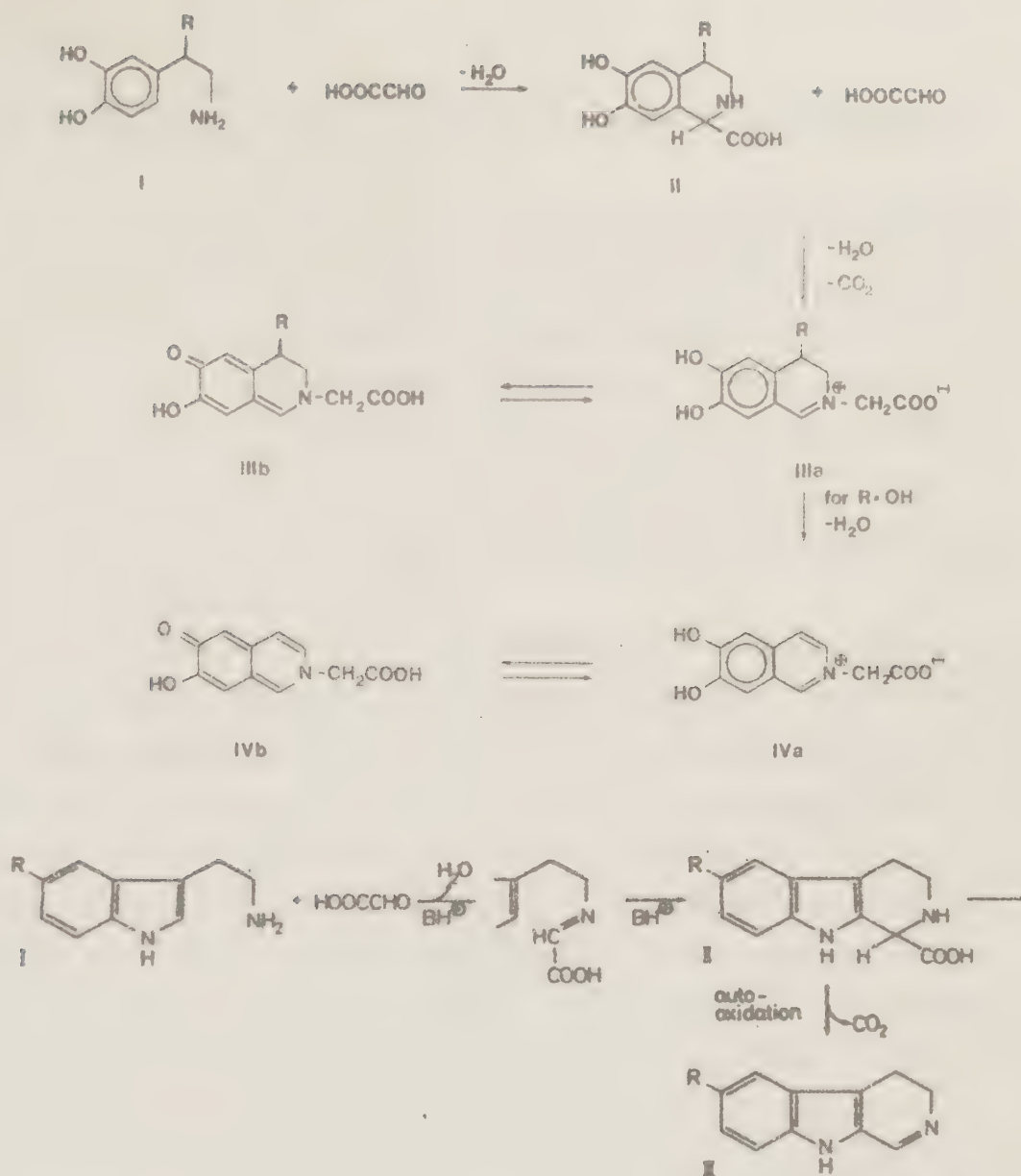


Figure 2. Sequence of reactions between catecholamines (Figure a), indolylethylamines (Figure b) and FA. R = H or CH₃ in Figure a. In Figure b; R = H for tryptamine; R = OH for 5-hydroxytryptamine. For further explanations, see text. (Reproduced from Björklund et al. 1973a).

A



B

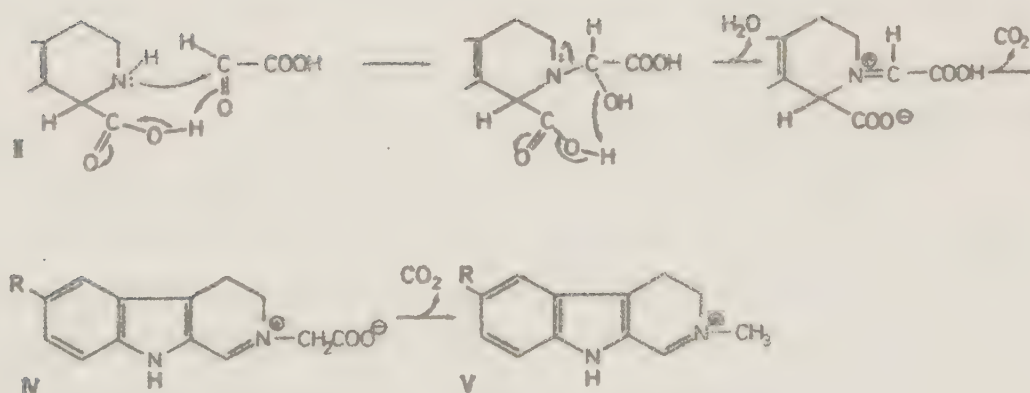


Figure 3. Sequence of reaction between catecholamines (Figure a), indolyethylamines (Figure b) and GA. In Figure a; R = H for DA; R = OH for NA. In Figure b; R = H for tryptamine; R = OH for 5-hydroxytryptamine. For further explanations, see text. (Reproduced from Björklund et al. 1973a; Lindvall et al. 1974).

by characteristic spectral changes (Björklund et al. 1968a, 1975; see below), the DA-fluorophore cannot react this way.

In the case of β -(3-indolyl)-ethylamines, such as tryptamine (T) and 5-hydroxytryptamine, the Pictet-Spengler reaction, which is acid-catalyzed results in the formation of weakly or non-fluorescent tetrahydro- β -carboline (II in Fig. 2b). The fluorophore formation takes place in a second step either via autoxidation to yield the dihydro- β -carboline (III in Fig. 2b) or in an acid catalyzed reaction with another FA molecule to form 2-methyl-dihydro- β -carboline (IV in Fig. 2b). The fluorophore forming reactions between FA and β -phenylethylamines and β -(3-indol)-ethylamines thus show great similarities.

The formation of fluorophores from β -phenylethylamines and β -(3-indolyl)-ethylamines in the GA histofluorescence method proceeds in two steps, which are analogous to those described for the FA histofluorescence method (Björklund et al. 1972b, Lindvall et al. 1974, Svensson et al. 1975). First, the β -phenylethylamines (DA and NA) and the β -(3-indolyl)-ethylamines (T and 5-HT) react with GA in an acid-catalyzed Pictet-Spengler cyclization, yielding tetrahydroisoquinoline-1-carboxylic acids (II in Fig. 3a) and tetrahydro- β -carboline-1-carboxylic acids (II in Fig. 3b). The tetrahydro-derivatives are then transformed into fluorophores via an autoxidative decarboxylation to dihydroisoquinolines (II and V in Fig. 2a; R=H) and dihydro- β -carboline (III in Fig. 3b). Alternatively, fluorophores are formed via a second intramolecularly acid-catalyzed reaction with GA to 2-carboxymethyl-dihydroisoquinolines (III in Fig. 3a) and 2-carboxymethyl-dihydro- β -carboline (IV in Fig. 3b). The 2-carboxymethyl-fluorophores may undergo a further decarboxylation to the 2-methyl-dihydroderivatives (2-methyl-dihydroisoquinolines, II and V in Fig. 2a; R = CH₃ or 2-methyl-dihydro- β -carboline, V in Fig. 3b).

The DA and NA fluorophores formed in the GA reaction exhibit a pH-dependent tautomerism, which is similar to that obtained for the DA and NA fluorophores in the reaction with FA (Lindvall et al. 1974). At neutral pH the fluorophores exist in a quinoidal form (IIIb and IVb in Fig. 3a) and in an acid milieu they exist in a non-quinoidal form (IIIa and IVa in Fig. 3a). As in the FA reaction, the 4-hydroxy group of the NA-fluorophore can be splitted off with acid (exposure to HCl-vapour) and thereby converted into a fully aromatic compound (2-carboxymethyl-6,7-dihydroisoquinoline, IV in Fig. 3a).

Aldehyde fluorescence methods for ontogenetic studies

Ontogenetic studies of the development of CA and 5-HT systems in the CNS (a detailed reference list is given by Seiger 1977) and PNS (de Champlain et al. 1970, Read and Burnstock 1969) have been performed with the Falck-Hillarp method and the GA-Vibratome method (Lidov et al. 1978, Yamamoto et al. 1977). However, a detailed study of these systems has been hampered by shortcomings of available methods. During ontogenesis parts of the CA and 5-HT-storing systems contain very low amounts of respective amine. The Falck-Hillarp method does not possess sufficient sensitivity for demonstration of amine storage sites during development. Furthermore, the formed fluorophores diffuse easily as observed in the fluorescence picture. The diffuse fluorescence might also be caused by inefficient drying, since embryological and neonatal material have a high water content.

Several attempts have been made to increase the number of fluorescent terminal detectable in the microscope. The most commonly used procedures are (a) pharmacological pretreatment of the pregnant animal or the young rats with a MAO-inhibitor (Olson and Seiger 1972, Seiger and Olson 1973) or α -methyl-NA (Lidov et al. 1978) or (b) incubation of the tissue in NA or α -methyl-NA containing solutions (Read and Burnstock 1969, de Champlain et al. 1970). The specimens are then processed according to the Falck-Hillarp method. The effects seen after either way of pretreatment are twofold: increased amount of detected nerve fibers and increased fluorescence intensity.

The advantages of using the more sensitive reagent GA, which were obtained in adult animals for the demonstration of CAs in the CNS, could not be achieved in ontogenetic material. As already mentioned, GA is a poor fixative when applied on adult brains. The soft consistency, probably due to too high a water content, in the brains of fetal and young animals makes sectioning on the Vibratome instrument less suitable for such material.

Objectives of the present study

When this study was initiated the only histofluorescence method with sufficient sensitivity for the visualization of all central CA neuron systems was the GA or combined GA-FA Vibratome procedures. There was thus a great need for alternative methods with the same sensitivity but without the practical drawbacks of the Vibratome methods. For many reasons the most attractive mode of application of the histofluorescence methods is the freeze-drying, paraffin sectioning technique. Our initial attempts to apply the GA method to this technique was unsuccessful, probably because

of the poor penetration of the reagent into tissue blocks. For this reason the subsequent attempts were directed towards improvements of the fluorescence yield in the FA method. Our interest was then focused on certain metal ions for two main reasons: (a) Magnesium ions are known to inhibit the release of CAs from their neuronal storage sites (Boullin 1967, Kirpekar and Misu 1967, Farnebo 1971). This raised the possibility that magnesium ions might prevent the dislocation of amines (and fluorophores) during processing of the tissue, thereby increasing the sensitivity and precision of the method. (b) Magnesium and aluminum ions are known to be so-called Lewis acids (Pearson 1968a, b, Heslop and Jones 1976), suggesting that they could be used as acid catalysts in the FA reaction.

In the present thesis the usefulness of magnesium and aluminum ions as fluorescence promoting agents in the FA method has been studied in detail. On basis of these results several combined FA-magnesium and FA-aluminum procedures have been designed for application on freeze-dried, paraffin embedded material, and cryostat or Vibratome sections. During the course of this study it was discovered that aluminum was potent in promoting a fluorophore-forming reaction between FA and some tertiary and N-acetylated indolamines (e.g. melatonin). The further study of this reaction has led to the discovery of a principally new type of fluorophore-forming reaction for indolamines under histochemical conditions.

RESULTS

This section first summarizes our studies on the importance of various reaction parameters on the quality of the fluorescence picture in the different magnesium and aluminum methods. The findings have been used to design optimal procedures, for visualization of CAs and 5-HT in tissue (see Recommended procedures). Finally, this section gives an account of the protein model experiments, which have been used to study, in more detail, the action of aluminum and magnesium ions in the fluorophore forming reactions.

Freeze-drying and paraffin embedding

Magnesium ions (Paper I)

Effect of hypertonicity. The hypertonic MgSO_4 -containing perfusion solution appeared to have a dehydrating effect on the tissue as revealed by neuronal shrinkage artefacts. Perfusion with a hypertonic solution has previously been shown to improve the fluorescence picture in the Falck-Hillarp method (Fuxe et al. 1968, Hökfelt and Fuxe 1969). However, the positive effects of the magnesium ion-containing solutions could not be explained on basis of hypertonicity alone. Thus, replacement of MgSO_4 with equimolar concentrations of NaCl , Na_2SO_4 , or sucrose caused only marginal improvements of the fluorescence picture in comparison with parallelly processed unperfused tissue. Furthermore, it was found that higher concentrations of NaCl , Na_2SO_4 or sucrose in the perfusion solution had a negative effect.

Effects of FA- and GA-containing solutions. Perfusion with an isotonic buffer caused only marginal improvements of the fluorescence picture, and addition of 2% GA to the perfusion buffer had no positive effect. A clear improvement was, however, seen when the animals were perfused with a FA-containing solution (1%, 4% or 10%; pH 7.0). The best results were obtained with a 4% FA solution.

Effect of high concentrations of magnesium ions. The addition of MgSO_4 to the perfusion solution had a marked positive effect on the fluorescence picture, i.e. higher fluorescence intensity and less diffusion of the fluorophores in the NA and DA neuronal systems. Five concentrations of MgSO_4 (5, 10, 20, 30 and 40 g/150 ml perfusion fluid) were tested either alone or with 1% or 4% FA added. The highest

concentrations gave clearly the best results. Thus, several catecholaminergic fiber systems, which could not be demonstrated with the original Falck-Hillarp method were detected. This fluorescence picture was further somewhat improved by the addition of 4% FA. Increasing concentrations of MgSO_4 reduced the pH of the perfusion solution (5g MgSO_4 reduced pH to 6.5 and 40 g MgSO_4 reduced pH to 5.5). However, even when the pH was kept constant the positive effects of MgSO_4 were still evident. Two other magnesium salts, MgCl_2 and $\text{Mg}(\text{CH}_3\text{COO})_2$, were tested at concentrations equimolar to those for MgSO_4 . These other magnesium salts were, however, clearly inferior to MgSO_4 as fluorescence promoting agents, although MgCl_2 was equal to MgSO_4 at the lowest concentration tested.

Effect of pH. As it is known that the fluorophore forming reactions between FA and biogenic monoamines are catalyzed by acids and since pH was reduced in solutions where MgSO_4 had been added, it was of interest to find out if the positive effects were due to acid-catalysis of MgSO_4 or to other properties of the magnesium ion. Perfusion with isotonic, magnesium-free buffers, where pH had been reduced (7, 6, 5, 4 and 3) did not improve the fluorescence picture. With 4% FA present in the perfusion solution there was a slight improvement of the fluorescence picture with decreasing pH. Perfusions with the most acid solutions caused diffusion of the fluorophores (which was most obvious in the solutions without FA) and a disturbing deterioration of the tissue. Perfusion with optimal MgSO_4 concentration, with or without 4% FA added, at various pH (5.5, 5, 4, 5, 4, and 3) showed that the fluorescence picture was improved in parallel with decreasing pH. Thus, a brilliant fluorescence picture with minimal diffusion was obtained at pH 4.5, 4 and 3. The lowest pH caused disturbing tissue damage (see also above). With low concentrations of MgSO_4 (5g/150 ml perfusion solution), the fluorescence picture was markedly better if pH 5.5 (the pH obtained by dissolving 40 g MgSO_4 /150 ml buffer) was used instead of pH 6.5 (the pH obtained by dissolving 5 g MgSO_4 /150 ml buffer). Comparison of the fluorescence pictures obtained by low and high MgSO_4 concentrations at the same pH (5.5) clearly favoured the high MgSO_4 concentrations.

Effect of GA and FA in the MgSO_4 -containing solution. Several different concentrations of the reagents, alone or in combination, in the MgSO_4 -containing buffer were tested. The best results were obtained with a) 4% FA plus 40 g MgSO_4 or with b) 0.5% FA plus 2% GA and 40 g MgSO_4 in the perfusion solution (pH 4-5).

Effect of a pre-perfusion. In contrast to the ALFA procedures (see below) such a step was not necessary with magnesium-containing perfusion solutions since this perfusate did not cause intravascular precipitation. A minor improvement of the fluorescence picture was, however, obtained by pre-perfusion with an acid buffer, e.g., brighter fluorescence intensity and improved morphology due to a reduction of the shrinkage artefacts (Lorén et al. 1977).

Aluminum ions (Paper III and IV)

The possibility that the positive effects of magnesium ions could be due to their acid properties focused the interest upon other metal ions with Lewis acid properties (for further comments see discussion). Since the magnesium ion is a rather strong Lewis acid, it was of interest to test the fluorescence promoting effects of weaker as well as stronger Lewis acids. The sodium ion is a weaker Lewis acid than the magnesium ion. It has previously been shown (see above) that sodium ions are much less efficient than magnesium ions in improving the fluorescence picture. The aluminum ion, on the other hand, which is a stronger Lewis acid than the magnesium ion, gave a markedly improved fluorescence picture also in comparison with that observed with the magnesium ion. It was found, however, that the $\text{Al}_2(\text{SO}_4)_3$ -containing solutions had to be perfused in a different way from the MgSO_4 -containing ones. Aluminum sulphate causes precipitation in the blood, which results in intravascular occlusion. Thus, the pre-perfusion step is needed to rinse the blood out of the vessels. Perfusion by means of a syringe proved to give variable results, with uneven distribution of the perfusate causing an uneven fluorescence picture and poor reproducibility. Therefore, a pressurized perfusion system was designed, which made it possible to introduce the pre-perfusion and the main perfusion solutions under strictly controlled conditions. The perfusion can be carried out under a controlled, high pressure, and the pressure can be varied for the different perfusion steps, i.e. the pre-perfusion and the main perfusion. A series of experiments were carried out to test the optimal volume of perfusate and concentrations of aluminum sulphate and reagents, as well as the best type of salt in the perfusion solutions. Similar to the results obtained for magnesium salts, aluminum chloride was found to be inferior to aluminum sulphate in its fluorescence-promoting effects. The optimal fluorescence picture was obtained with 2% FA and 10-20 g $\text{Al}_2(\text{SO}_4)_3 \times 18 \text{H}_2\text{O}$

dissolved per 100 ml buffer in the main perfusion solution (pH adjusted to about 3.8 by means of 0.38 g $\text{Na}_2\text{B}_4\text{O}_7 \times 10 \text{H}_2\text{O}$ per 100 ml buffer) and with 2% GA in the pre-perfusion solution. With the optimal procedure the central DA neuronal systems were detected with at least the same sensitivity as obtained with the GA-Vibratome method, while the central NA neuronal systems were demonstrated with a higher sensitivity.

The fluorescence picture will be briefly commented. Certain DA-containing systems, e.g. the incerto-hypothalamic system, the dendrites of the substantia nigra neurons, and the projections to the prefrontal cortex and the anterior cingulate cortex systems contain very low amounts of transmitter, and have therefore previously been visualized only with the GA-Vibratome method (Lindvall and Björklund 1978). Another system, which might be difficult to visualize with the standard Falck-Hillarp method, is the tubero-hypophyseal system (Björklund et al. 1974). All these systems are demonstrated with a sensitivity similar to that observed in the GA-Vibratome method. Furthermore, the ALFA method is more suitable for demonstration of central CA nerve cell bodies than the GA-Vibratome method because of a better preservation of these structures. Elimination of the reagents results in a marginal decrease of the fluorescence intensity in certain low amine-containing systems. This shows that the fluorescence-promoting effects are exerted by the aluminum ions, as was the case for the magnesium ions in the Mg-FA-GA method. GA has to be introduced via the pre-perfusion solution, since its presence in the aluminum-containing perfusion solution resulted in a fluorescence picture, which was much inferior to that obtained with the standard Falck-Hillarp method.

This variant of the ALFA method was also successfully applied for the neuronal and non-neuronal demonstrations of CA:s and 5-HT in ganglia and endocrine and visceral organs. Certain adrenergic nerves of the ovary, pancreas, the gastrointestinal tract, thyroid gland, which are not or only poorly detectable with the standard Falck-Hillarp method, were clearly demonstrated. SIF cells, mast cells, enterochromaffin cells and APUD cells (visuable after i.p. administration of L-Dopa) exhibit a bright fluorescence intensity without any signs of diffusion. Thus, all these storage sites (nerves and cells) can be studied in more detail than has previously been the case for freeze-dried and paraffin embedded tissue samples (see also Sundler et al. 1980, Ahrén et al. 1981).

Methods for ontogenetic studies (Paper V)

The animals used are normally pretreated with the MAO-inhibitor pargyline or with α -methyl-NA, which is accumulated in CA neurons (for further comments, see Pharmacological pretreatments below).

Magnesium ions

Perfusion of rat embryos and young rats with the Mg-FA-GA-containing perfusion solution markedly improved the fluorescence picture compared to unperfused animals processed in parallel (Lorén et al. 1976). The fluorescence picture was somewhat further improved if the animals first were preperfused with an acid buffer (pH 4.5). The perfusions were carried out by means of a syringe and the tissue samples were then further processed according to the Falck-Hillarp method (Björklund et al. 1972a, Lorén et al. 1976).

In addition to the higher sensitivity, the CA-systems in the developing brain were demonstrated with minimal diffusion and with an increased reproducibility. Neuronal structures were demonstrated at earlier stages of development than seen in unperfused animals treated in parallel (compare with Loizou 1972 and Seiger and Olson 1973). The high sensitivity of the Mg-FA-GA-method was observed, e.g., in the hippocampus and in the cerebellar and cerebral cortices where the locus coeruleus NA terminals were well demonstrable at birth (see also Levitt and Moore 1979, Loy and Moore 1979). The DA terminal system in the prefrontal cortex was also shown.

In an earlier report, animals, that were not pretreated with a MAO-inhibitor, were used (Lorén et al. 1976, see above). Pretreatment of the animals with an MAO-inhibitor prior to perfusion and/or dissection is advantageous for CA and, in particular, for 5-HT visualization in neonates. Only the cell bodies and the proximal processes of 5-HT neurones are visible in non-pretreated animals (Olson and Seiger 1972, Seiger and Olson 1973). After MAO-inhibition these parts of the neuron were visualized in more detail in the Mg-FA-GA-method than with the standard Falck-Hillarp method. However, as was also the case after aluminum perfusion (see below), the sensitivity was too low to allow a detailed demonstration of 5-HT-containing terminals in the developing rat brain.

Aluminum ions

In comparison with the result obtained with the Mg-FA-GA-method, the fluorescence picture obtained with the ALFA method was more sharply defined, more reproducible and the fluorescence intensity was much brighter. The reproducibility was markedly increased by the use of a room-tempered pre-perfusion with MgSO_4 and procain chloride added. The neurons were demonstrated to a fuller extent and no shrinkage artefacts were observed in animals processed according to the ALFA method. Pretreatment of the animals with α -methyl-NA, which is taken up into catecholaminergic neurons, led to a marked increase of their fluorescence intensity and allowed for a much more complete visualization of these neuron systems than is the case after administration of the MAO-inhibitor. This can be exemplified by the projection systems originating from locus caeruleus and substantia nigra, which showed a bright fluorescence after α -methyl-NA pretreatment but were only moderately fluorescent after administration of the MAO-inhibitor. After pretreatment with α -methyl-NA more details were visualized, such as dendrites, axon collaterals and terminal arrangements.

Although a minor uptake into non-monoaminergic neurons cannot be ruled out absolutely, the vast majority of innervation made visible after the administration of α -methyl-NA is catecholaminergic. This can be inferred from several observations. a) The distribution of neurons and their processes after α -methyl-NA treatment conforms well with that of CA innervation in the adult. b) At least in the 6-day-old rat, lesions in the ascending CA bundle at the level of the mesencephalon with 6-OH-DA remove all cortical innervation visible after α -methyl-NA pretreatment (Lidov et al. 1978). c) Neonatal subcutaneous 6-OH-DA treatment abolishes all α -methyl-NA-accumulating fibers of presumed NA nature in the forebrain. d) Nomifensin, which is a potent and selective blocker of CA uptake into NA and DA neurons (Ehsanullah and Tumor 1977, Schacht, Levin and Backer 1977), blocks all accumulation of α -methyl-NA into axons and terminals in the forebrain. This is also seen if the animals are treated with α -methyl-p-tyrosine (which is a tyrosine hydroxylase inhibitor) prior to treatment with nomifensine and α -methyl-NA. After such combined treatment the presumed NA and DA terminal areas of the forebrain were non-fluorescent. The only unspecific uptake was observed in capillaries. However, this does not interfere with the interpretation of the microscopic picture. In the following some examples of the fluorescence picture obtained in animals pretreated with α -methyl-NA or a MAO-inhibitor will be given.

Cerebral cortex. The NA-containing nerve fibers and terminals in cortex of α -methyl-NA pretreated newborn rats were demonstrated with a very high sensitivity and precision. The amount of nerve fibers and terminals exceeded that described for the 6-day-old rat by Lidov et al. (1978), showing that the cortical NA innervation is extensive already at birth (see also Levitt and Moore 1979). Animals pretreated with a MAO-inhibitor showed a similar number of fibers although with much lower fluorescence intensity.

The DA innervation in the deep layers of prefrontal cortex (i.e. the anteromedial frontal cortex and the sulcal cortex) and in the perirhinal cortex was visualized already at birth, while the innervation of the anterior cingulate, the entorhinal and the piriform cortices appeared to be poorly developed. Most interestingly, an apparent dopaminergic hyperinnervation pattern was seen in the prefrontal cortex at birth. This hyperinnervation was most pronounced in the newborn rat and was reduced during the first postnatal week.

Hippocampus. In aluminum perfused brains, particularly after pretreatment with α -methyl-NA, the number of CA terminals was markedly increased but never reached the number seen in adult animals. The rostral hippocampus contained the densest CA innervation pattern, which slowly decreased in caudal direction. In the rostral part the densest innervation was seen in the dentate gyrus and in the supra- and infra-pyramidal zones of the CA1 and CA3 areas. The CA innervation of the caudal hippocampus was most pronounced in the dentate hilus and in the molecular layer of CA1 and CA3. The CA nerve fiber bundles – in the subiculum, in the fimbria-fornix and in the alveus-, which are projecting into hippocampus, were clearly demonstrated.

Another striking hyperinnervation patterns at birth was seen in the anterior septum and in the rostral hippocampal rudiment. This was most clearly seen after pretreatment with α -methyl-NA. As was also the case in the prefrontal cortex (see above) this hyperinnervation pattern was reduced during the first postnatal week.

Cerebellum. Pretreatment of the animals with the MAO-inhibitor was sufficient for demonstration of part of the CA nerve fibers and terminal projections into the cerebellum. However, if α -methyl-NA was used instead of pargyline, the fluorescence intensity in and number of CA-containing structures were markedly increased. A third example of a transient hyperinnervation was observed in the cerebellum during the second (day 7-14) postnatal week.

Cell bodies. In aluminum perfused brains a detailed visualization of CA nerve cell bodies, their dendrites and axonal processes was obtained and the fluorescence intensity was markedly increased if the animals had been pretreated with α -methyl-NA. This kind of pre-treatment also demonstrated the CA-producing periglomerular cells and their processes in the olfactory bulb, which cannot be visualized with aldehyde fluorescence histochemistry without loading with exogenous CA:s (see Halász et al. 1977).

Indolamine visualization (Paper I, III and V)

For demonstration of 5-HT neurons in developing and adult rat brain, the animals were pretreated with pargyline or L-tryptophan, chloralhydrat and pargyline (see Aghajanian et al. 1973). In rat brains processed according to the Mg-FA-GA-method all 5-HT-containing nerve cell bodies are well visualized. The nerve cell bodies, their dendrites and axons as well as axon bundles are well demonstrated without signs of diffusion. They exhibit intense and bright yellow fluorescence, which fades rather rapidly when illuminated. Fluorescent terminals are demonstrable in, e.g., neocortex, hippocampus, globus pallidus, substantia nigra, superior colliculus, nc. interpeduncularis, and spinal cord (cf. Lorén et al. 1977).

When the rat brains were processed according to the ALFA method, the same neuronal components as described above (see the Mg-FA-GA-method) were demonstrated with the same brightness and precision. Also in these specimens they exhibited the characteristic fading of intraneuronal 5-HT. However, it is difficult to differentiate between CA:s and 5-HT in the ALFA method although the brightness of the fluorescence picture. In this method the CA:s exhibit a yellow fluorescence color. This is due to a spectral shift of the emission spectra for CA:s to longer wavelengths, i.e. similar to that recorded for 5-HT (see below). The best way to demonstrate 5-HT with either of these methods is by depletion of the CA systems, e.g., after lesions of CA nerve fiber bundles or after administration of a neurotoxic agent such as 6-OH-DA.

The main advantage of these procedures is the sharpness of the fluorescence picture showing 5-HT-containing cell bodies, dendrites, axons and varicose terminals with a richness in detail that is superior to other available FA or GA procedures. However, these methods do not improve the visualization of 5-HT neurons to the same extent as seen for the CA:s.

Cryostat techniques

The most successful cryostat methods for monoamine fluorescence histochemistry have been based upon perfusion of tissue before sectioning and exposure of the sections to the reagents FA and/or GA. These principles were therefore adopted in the present study. The following perfusion and immersion solutions were tested a) the most optimal perfusion solutions used in the Mg-FA-GA and ALFA methods (see above) b) perfusions with the respective magnesium and aluminum sulphate with or without 4% FA added c) immersion solutions made from the respective magnesium or aluminum sulphate or 2% GA solution. Unperfused tissue was also used; these sections were immersed in an immersion solution containing either magnesium or aluminum ions. To clarify the effects of the different ions some sections were processed in parallel but with the immersion step omitted. Other parameters of critical importance for the cryostat techniques, such as mode of freezing of the tissue, sectioning temperature, and drying procedures, were also evaluated. FA vapour treatment was generally used to develop the fluorophores.

Magnesium ions (Paper II)

Immersion of non-perfused tissue sections in an ice-cold solution of MgSO_4 or MgCl_2 improved the fluorescence picture compared with sections treated in parallel, but with the immersion step omitted. Perfusion with a MgSO_4 -containing solution with or without 4% FA added, markedly improved the fluorescence picture, and there was a further marginal improvement by an additional immersion in MgSO_4 or MgCl_2 . The best result was obtained if the animals were perfused with the Mg-FA-GA solution. A short immersion of the sections in a 2% GA solution before drying and FA vapour treatment optimized the result.

The technical steps of special critical importance are the freezing procedure and the temperature of the cryostat during sectioning. In order to avoid freezing artefacts the tissue samples were either frozen in the cryostat chamber or in powdered dry ice. The latter procedure is advantageous, since freezing occurs more rapidly in the powdered dry ice. The temperature of the cryostat ought to

be between -25 and -30°C . Higher temperatures cause diffusion and disturbances of the fluorescence picture. At sectioning temperatures below -25 to -30°C the tissue morphology is disturbed by cracking artefacts. At these temperatures serial sectioning is difficult to perform, which is not the case with a higher sectioning temperature. A short drying of the sections (5-20 min) under the warm air-stream generated from a hair-dryer, before the sections were further dried (0.5-24 h) in vacuo over P_2O_5 in a dessicator, proved to be an adequate drying procedure.

Comments of the fluorescence picture. The results obtained with the most optimal procedure (see Recommended procedure) have been characterized in some detail. All known NA and DA neuronal systems are well demonstrable in all areas of the brain. The only exceptions are the incerto-hypothalamic DA system and the DA innervation of the anterior cingulate cortex, which indicates that these terminal systems are beyond the sensitivity for visualization with this method. The NA and DA nerve cell bodies, dendrites, axons and terminal areas are demonstrated with a high precision, i.e. high fluorescence intensity and with no diffusion. The Mg-FA-GA method has thus a markedly higher sensitivity than the original Falck-Hillarp method for the demonstration of central CA neurons. This is not the case for 5-HT visualization, which is poor with this procedure and below the capacity of the original Falck-Hillarp method.

Aluminum ions (Paper III and IV)

The ALFA method for cryostat sections was applied both on CNS and as well as endocrine and visceral organs using different parameters depending on the tissue. Specimens from both perfused and non-perfused animals were examined, different temperatures of the cryostat chamber (i.e. the sectioning temperature) were tested, as well as immersion of the freshly cut sections in an aluminum ion containing buffer.

The best results in CNS tissue was obtained with the same perfusion solutions as those used for freeze-dried and paraffin-embedded specimens (i.e. a pre-perfusion solution consisting of 150 ml of a Tyrode's buffer or a 2% GA solution, and a main perfusion solution consisting of 300 ml buffer with 30-60 g $\text{Al}_2(\text{SO}_4)_3 \times 18\text{H}_2\text{O}$ 1.14 g $\text{Na}_2\text{B}_4\text{O}_7 \times 10\text{H}_2\text{O}$ and 2% FA added). The best results obtained in peripheral neurons as well as in non-neuronal storage sites were obtained with the same

perfusion solutions as those used for CNS tissue, or with a pre-perfusion consisting of 150 ml Tyrode's buffer followed by perfusion with 300-450 ml Tyrode's buffer with $\text{Al}_2(\text{SO}_4)_3$, $\text{Na}_2\text{B}_4\text{O}_7$ and 4% FA added. The perfusion steps were carried out by means of the pressurized perfusion system designed for the ALFA method (see above in section on freeze-dried and paraffin embedded tissue). Immersion of sections obtained from perfused tissue did not further improve the fluorescence picture, rather, the immersion step reduced the quality of the result. To improve the tissue morphology, especially in sections obtained from CNS tissue, 2.5-5% sucrose was added to the perfusion solutions. Non-perfused tissue can in some cases be the only choice for demonstration of CA:s and 5-HT in neuronal as well as non-neuronal storage sites. In the present study we used non-perfused tissue samples from visceral organs. Immersion for 30-60 secs in an aluminum ion containing solution ($1.7\text{-}2.5\text{ g Al}_2(\text{SO}_4)_3 \times 18\text{ H}_2\text{O}$ dissolved in 100 ml Tyrode's buffer) markedly improved the fluorescence picture compared to sections parallelly treated but with the immersion step omitted. This variant of the ALFA method proved to be quite useful although its sensitivity is below that seen after perfusion.

The sectioning temperature, i.e. the temperature of the cryostat chamber is (as previously mentioned) of critical importance. The best result is reached if the sections are cut at -25 to -30°C . Sectioning at -15°C is possible for the demonstration of NA and DA in the PNS, but useless for demonstration of CA:s in the CNS. As is the case with the sectioning temperature, the other steps used in the ALFA cryostat procedure (i.e. freezing of the tissue in powdered dry ice, the drying procedures and the FA vapour treatment) are the same as those employed in the Mg-FA-GA procedure. However, the ALFA method gives clearly better results than the Mg-FA-GA method.

Comments on the fluorescence picture. Only CA neurons are well demonstrated in CNS tissue with the ALFA method. This is the case also for other cryostat methods published. The 5-HT neurons are only weakly fluorescent, even after MAO-inhibition and tryptophan loading. The fluorescence picture seen in the ALFA method represents a further improvement over that seen in the Mg-FA-GA method. The fluorescence intensity, the sharpness of the fluorescent nerve fibers, and the amount of nerve fibers are clearly enhanced. Thus, all NA and DA systems can be demonstrated in more detail with the ALFA method. Even systems with low amine content, such as the

incerto-hypothalamic and anterior cingulate cortex DA systems can be visualized. Nevertheless, the ALFA cryostat method does not reach that quality and reproducibility, which is seen when the ALFA and Mg-FA-GA methods are applied on the freeze-drying, paraffin embedding technique as well as the Vibratome technique.

For the demonstration of CA:s and 5-HT in endocrine and visceral organs, perfusion of the tissues proved to be of advantage. The sensitivity in detection of peripheral storage sites was at least equal to that obtained with the standard Falck-Hillarp method, and not far from that seen in specimens parallelly processed with freeze-drying and paraffin embedding. Unperfused tissue samples of visceral organs show a remarkable improvement of the fluorescence picture if these sections were immersed in an aluminum ion containing solution compared to sections processed in parallel but with the immersion step omitted.

Sectioning at a low temperature, -20 to -25°C , was favourable for optimal fluorescence demonstration, but the drawbacks with the low temperatures were the same in this case as previously described for CNS tissue samples. Thus, if sectioning was performed at about -15°C a small decrease of the fluorescence picture was present but instead an improved section morphology and easier serial sectioning was achieved.

Vibratome techniques

The previously developed Vibratome procedures are based on perfusion with either of the reagents FA or GA; the latter reagent being the hitherto most sensitive one for demonstration of central CA:s. The GA-Vibratome method is technically somewhat difficult to handle since GA is a bad fixative, which makes sectioning difficult (unless experience of this kind of sectioning is at hand) and does not allow serial sectioning. FA is a good fixative and sectioning is thus much easier in the FA-Vibratome method.

Magnesium ions (Paper II)

Magnesium ions were tested alone and in combination with FA and GA. The best result with respect to the fluorescence picture and section quality are obtained if the animals are perfused with 150 ml of a 4% FA solution with 2-5 g MgSO_4 added. The freshly cut sections are immersed for about 2 min in a magnesium

ion containing buffer (5 g $\text{MgSO}_4 \times 7 \text{H}_2\text{O}$ or equimolar concentration of MgCl_2 dissolved per 150 ml Tyrode's or Krebs-Ringer bicarbonate (KRB) buffer). The same concentrations of magnesium ions added to various steps of the GA-Vibratome method give only marginal improvements of the fluorescence picture; sectioning is, however, still difficult. Thus, the Mg-FA-method applied to Vibratome sections seems to be the best variant.

Comments of the fluorescence picture. The results achieved with the Mg-FA-method (not the Mg-FA-GA-method since the reagent GA is excluded in this Vibratome procedure) demonstrates all NA and DA neuronal systems with a high sensitivity and precision. The only exceptions are the systems with low DA content, such as the incerto-hypothalamic and anterior cingulate cortex systems. Another system with low amine content – the prefrontal DA terminal system – is well visualized and with a high precision. The NA and DA cell bodies, dendrites, axons, axon bundles and terminal areas have a high fluorescence intensity, and outline without diffusion. This Mg-FA method has in our hands a higher sensitivity than the FA-Vibratome method (Hökfelt and Ljungdahl, 1972) and the Mg-FA-GA method applied on cryostat sections and it has almost the same sensitivity as the Mg-FA-GA method applied on freeze-dried and paraffin embedded tissue, whereas it is inferior to the GA-Vibratome method. The Mg-FA-Vibratome method is therefore suitable for demonstration of most central NA and DA systems.

Aluminum ions (Paper III)

The ALFA histofluorescence procedure applied to Vibratome sections is performed by replacing the magnesium ions in the immersion bath with aluminum ions, while the perfusion solution consists of a 4% FA solution without aluminum ions added. The animals are perfused with 300 ml of an ice-cold 4% FA solution. The freshly cut sections are immersed for 15-30 sec in an aluminum ion containing solution (1.7-2.5 g $\text{Al}_2(\text{SO}_4)_3 \times 18 \text{H}_2\text{O}$ dissolved in 150 ml of a Tyrode's or KRB buffer).

Comments of the fluorescence picture. All central NA and DA neurons are demonstrated with a high sensitivity and precision. Also the low DA-containing systems in the prefrontal cortex, the incerto-hypothalamic and anterior cingulate cortex are clearly demonstrated. The NA and DA neuronal systems are visualized with a sensitivity similar to that seen in the ALFA and Mg-FA-GA methods

applied on freeze-dried and paraffin embedded material and also to that seen in the GA-Vibratome method. Thus, exchanging the magnesium ions for aluminum ions gives a clearly more sensitive Vibratome procedure.

A further improvement of the Mg-FA and ALFA Vibratome methods is present if the animals under a longer period are perfused with larger volumes of the 4% FA solution, probably due to better fixation of NA and DA in the tissue. This also helps further fixation of the brains, which then are more easy to section.

5-HT visualization in Vibratome sections is rather poor even after pharmacological pretreatment. This is the case for both the Mg-FA and the ALFA-Vibratome methods as well as the FA- and GA-Vibratome methods.

Whole-mounts

Aluminum and magnesium ions (Paper IV)

Comments of the fluorescence picture. Sufficiently thin tissue preparations can be stretched onto glass microscope slides and then processed for fluorescence microscopy (so-called whole-mounts or stretch preparations). The best results are obtained if the animals are perfused either according to the optimal Mg-FA-GA procedure (unpublished observations) or the ALFA procedure as described for the freeze-dried and paraffin embedded specimens or for the cryostat sections. The tissue samples are then stretched on microscope slides, dried first under the warm air-stream from a hair-dryer, and then over P_2O_5 in vacuo in a desiccator, before they are exposed to FA vapour according to the standard Falck-Hillarp method. Specimens from animals perfused according to the ALFA method showed the best fluorescence picture, with a higher fluorescence intensity, good sharpness of delicate details, lack of diffusion, and a high reproducibility.

In unperfused tissue the fluorescence picture is markedly improved if the specimens are immersed in an aluminum ion containing solution (1.7-2.5 g $Al_2(SO_4)_3 \times 18 H_2O$ dissolved in 100 ml of a Tyrode's or KRB buffer) for 1-5 min. After immersion, the specimens are stretched on microscope slides and processed as described above for perfused tissue. Also in this case the fluorescence picture is markedly improved (brighter fluorescence intensity, higher reproducibility and no diffusion) as compared to the standard Falck-Hillarp method described for whole-mounts. Thus, delicate nerve fibers are visualized with a

sensitivity similar to that obtained with the most sensitive techniques previously developed for whole-mounts, i.e. those based on reaction with GA (Furness and Costa 1975, Lindvall and Björklund 1974, Waris and Partanen 1975), and the above described variants of the Mg-FA-GA and ALFA methods. Somewhat surprisingly, only small improvements of the fluorescence picture were observed after immersion of unperfused tissue in a magnesium ion-containing immersion solution compared to specimens treated in parallel but with the immersion step omitted (i.e. a standard Falck-Hillarp preparation).

Chemistry

Fluorophore formation from primary and secondary CA:s and IA:s (Paper VI)

The action of aluminum and magnesium ions on the formation of fluorophores in the FA reaction and their spectral characteristics was studied in protein models. Fluorescence yields. The presence of aluminum ions in the protein models enhanced the fluorescence yield of DA (twice) and T (20 times) after FA vapour treatment while no effect was observed in NA- and 5-HT-containing droplet models. Magnesium ions increased the fluorescence yield from T (5 times), while DA was unaffected and NA and 5-HT showed a decreased fluorescence yield. Thus, there is obviously a clear discrepancy between the fluorescence intensities recorded in models and those in tissue, since the fluorescence yields from intraneuronal DA and NA was 3-4 times higher after aluminum perfusion than after treatment according to the standard Falck-Hillarp method.

Concentration dependance. Maximal fluorescence yield from T was obtained at 5-10 mM of $\text{Al}_2(\text{SO}_4)_3$. The fluorescence yield was then 4-5 times higher than the maximal value obtained in MgSO_4 -containing protein droplets even if the concentration of MgSO_4 was raised 10-20 times.

pH dependence. Addition of aluminum, magnesium or sodium salts to the model solutions resulted in a reduction of pH, which paralleled their strength as Lewis acids. Thus, the most pronounced reduction of pH was seen with aluminum ions, somewhat less for magnesium and insignificant for sodium, the latter being a weak Lewis acid. Optimal pH was found to be about 3.8 (adjusted by $\text{Al}_2(\text{SO}_4)_3$ itself when added to the protein solution) for the aluminum-catalyzed FA reaction; changing of the pH caused a reduction of the fluorescence yield. This was not

the case for MgSO_4 , which had a wider pH scale (pH between 3.5-5.5) for production of optimal fluorescence yield. At pH 3.8 the fluorescence yield of $\text{Al}_2(\text{SO}_4)_3$ was about 4 times higher than that obtained for MgSO_4 at equal pH and at equimolar concentration.

Effect on synthetic fluorophores. The mechanisms underlying the fluorescence-enhancing action of the three Lewis acids were further characterized by comparing the effects of equal concentrations (10mM) of the metal salts on the fluorescence yield obtained from the synthetic FA-induced fluorophores from tryptamine (3,4-dihydro- β -carboline and 2-methyl-3,4-dihydro- β -carboline), with those observed on the tryptamine fluorescence in the FA reaction. Thus, $\text{Al}_2(\text{SO}_4)_3$ produced a 9-12 fold increase of the fluorescence yield both for tryptamine and for its synthetic fluorophores in the FA reaction; this increase was 5-7 fold if $\text{Al}_2(\text{SO}_4)_3$ was mixed with the synthetic fluorophores and if no FA reaction was performed. MgSO_4 caused a 2-3 fold increase of fluorescence intensity for both tryptamine derived fluorescence as well as of the synthetic fluorophores in the FA reaction. Na_2SO_4 had no significant effect in either case. This shows that the metal salts have qualitatively and quantitatively similar effects on the fluorescence intensities of the synthetic tryptamine fluorophores as well as on the tryptamine-derived fluorescence. These findings suggest that the fluorescence-promoting effects are primarily due to a direct interaction with the fluorophores formed in the FA reaction.

Spectral characteristics of the CA and 5-HT fluorophores in the ALFA reaction.

The emission spectra of NA and DA in aluminum ion containing droplets showed a maximum peak at about 510-520 nm, while that of 5-HT was at about 520-525 nm. The emission spectra of NA, DA and 5-HT recorded from cell bodies and terminals of the rat brain showed similar values. Thus, the color difference that exist between CA:s and 5-HT under salt-free conditions was almost abolished in the ALFA treated protein models and tissue samples. The excitation spectra of NA and DA in aluminum-containing droplets showed two peaks, one at about 350 nm and one at about 410 nm (the size of respective peak is pH dependent), while 5-HT had one major peak at 365 nm. Spectral recordings of NA, DA and 5-HT from cell bodies and terminals of the rat brain showed one peak at about 350 nm and the maximum peak at about 410 nm, while 5-HT only showed one peak at about 410 nm.

Fluorophore formation from tertiary and N-acetylated indolamines (Paper VII)

In paper VII three different ways are described for the visualization of

tertiary and N-acetylated indolamines. It is shown that GA, FA in combination with aluminum ions (the ALFA method) and trifluoroacetic acid anhydride (TFAA) are capable to form fluorescent compounds from these indolamines in histochemical protein models. In the present summary only the fluorophore formation in the ALFA method will be further commented.

Fluorescence yield. N-acetylated and tertiary indolamines did not yield any significant fluorescence in the standard FA reaction but became strongly fluorescent when enclosed in aluminum-containing droplets prior to the FA reaction. The fluorescence yield of melatonin was very low at a concentration of 1 or 10 mM $\text{Al}_2(\text{SO}_4)_3$ but increased (10-fold) dramatically if the $\text{Al}_2(\text{SO}_4)_3$ concentration was raised 10-fold i.e. 100 mM $\text{Al}_2(\text{SO}_4)_3$. This should be compared with the values obtained from T in similar experiments (see above). Furthermore, longer reaction times and a more humid FA vapour treatment also increased the fluorescence intensity from melatonin (about 3 times). At optimal reaction conditions for melatonin (100 mM $\text{Al}_2(\text{SO}_4)_3$; FA vapour treatment at $+80^\circ\text{C}$ for 1 h with 90% relative humidity) also primary CA:s (DA and NA), primary IA:s (T, 5-HT and 5-methoxy-T), N-acetylated DA and 5-HT, bufotenin (N,N-dimethyl-5-HT), tryptophan as well as 5-hydroxyindole acetic acid (5-HIAA) became strongly fluorescent. Most of these compounds had a higher fluorescence yield in the aluminum-catalyzed FA reaction than after standard FA treatment.

The reaction conditions necessary for fluorescence development from melatonin, T and 5-HIAA were further investigated. Both FA and aluminum ions were necessary for the fluorophore formation from melatonin and 5-HIAA. If the FA reaction was performed in neutral or acid environment, but with the aluminum ions excluded, no fluorescence was obtained from melatonin and 5-HIAA. In contrast, T became strongly fluorescent after reaction with FA both in a) an acid environment without aluminum ions and b) in aluminum ion containing droplets. No fluorescence was induced from melatonin and 5-HIAA when they were reacted with FA in models containing magnesium or sodium ions. The fluorescence enhancing effect of aluminum ions in the FA reaction with melatonin was not seen in the GA reaction, which might be explained by an unsuitable complex formation between the aluminum ions and the GA-melatonin fluorophore.

Spectral characteristics of the fluorophores formed from indolamines and related compounds in the ALFA reaction. The indole compounds tested emitted a yellow

fluorescence with the emission peak maximum varying between 505 and 550 nm. Based on their excitation spectra, the tested indole compounds were divided into four major groups. Group A (tryptophan and T) has a narrow peak at 370 nm. Group B (melatonin and N-acetyl 5-HT), which comprises N-acetylated compounds, has a peak maximum at 370 nm and two minor peaks at 340 nm and 445 nm or higher wave-lengths. Group C (bufotenin, 5-HT and 5-HIAA), which comprises the other 5-hydroxylated compounds, has a major peak of 375-380 nm, a rather high peak at 420-435 nm and a small peak at 320-330 nm. Group D (6-HT), which comprises a 6-hydroxylated compound, has a major peak at 440 nm and a low peak at 330 nm.

Tissue experiments. Rat brains and pineal organs were processed according to the ALFA method developed for freeze-drying and paraffin embedding, but with increasing concentrations of $\text{Al}_2(\text{SO}_4)_3$ (10-50 g $\text{Al}_2(\text{SO}_4)_3$ /100 ml buffer) in the perfusion solution and with a humid FA vapour treatment (90% humidity). The pineal gland showed a specific diffuse fluorescence, which had the spectral characteristics of 5-HT. The CA-containing structures of the brain had an intense fluorescence, which was weaker in the 5-HT neurons. At the highest concentrations tested, the fluorescence intensity and amount of CA fluorescence were clearly decreased.

N-acetylated indolamines have been claimed to occur in neurons and glia cells in the granule cell layer of the cerebellum, the spinal tract of the trigeminal roots and in the pontine reticular formation (Bubenik et al. 1974, 1976). These authors used an immunohistochemical method for demonstration of N-acetylated indolamines in the CNS. When carefully analyzed, no specific fluorescence was observed in ALFA treated material, that corresponded to the findings described by these authors.

Recommended procedures

Freeze-drying and paraffin embedding

f. The ALFA method: adult animals (Paper III and IV)

The animals are perfused by means of a pressurized perfusion system (described in detail in paper III). The perfusion must be performed under strictly controlled conditions.

A. Perfusion procedure. The animals are perfused in two steps, first with a pre-perfusion solution to rinse the blood out of the vessels (see above) and then with the main perfusion solution. The pre-perfusion solution consists of a) 150 ml of an ice-cold Tyrode's buffer (pH 7.2) or b) 150 ml of an ice-cold 2% GA solution (pH 7.0). The main perfusion (the aluminum perfusion) solution consists of 300 ml of an ice-cold Tyrode's buffer with a) 30-60 g $\text{Al}_2(\text{SO}_4) \times 18 \text{ H}_2\text{O}$, 1.14 g $\text{Na}_2\text{B}_4\text{O}_7 \times 10 \text{ H}_2\text{O}$ and 2% FA added (CNS, PNS, endocrine and visceral organs) b) 30-60 g $\text{Al}_2(\text{SO}_4)_3 \times 18 \text{ H}_2\text{O}$, 1.14 g $\text{Na}_2\text{B}_4\text{O}_7 \times 10 \text{ H}_2\text{O}$ with or without 1-4% FA added (PNS, endocrine and visceral organs). The pre-perfusion is kept at 0.6-0.8 bar/cm². When switching from the pre-perfusion to the aluminum perfusion, the perfusion pressure is gradually increased to about 2.0 bar/cm². The perfusion volume is 300 ml with clamp over the descending aorta (CNS tissue) and 450 ml without clamp (CNS - spinal cord, PNS, endocrine and visceral organs). The entire perfusion is completed within 30 min. Marked swelling of the neck and head region is a bad sign and signifies that intra-vascular precipitation has taken place. When using the GA-containing pre-perfusion solution swelling will also occur when the perfusion pressure is too high.

B. Freezing. The tissue pieces are rapidly dissected. The pieces are put on identification tags and wrapped in cotton gauze (only CNS tissue; this helps to keep the piece together after freeze-drying). The specimens are then rapidly frozen in a liquid propane-propylene mixture, cooled to the temperature of liquid nitrogen (see Björklund et al. 1972a). Frozen specimens can be stored in liquid nitrogen for prolonged periods.

C. Processing. The specimens are freeze-dried, preferably at a temperature of -35 to -40°C, treated with FA vapour and embedded in paraffin in vacuo according to the Falck-Hillarp method (for details of the procedures and equipment used in our

laboratory, see Björklund et al. 1972a). The FA vapour treatment is performed at $+80^{\circ}\text{C}$ for 1 h, using paraformaldehyde equilibrated in air of 50–70% relative humidity. For optimum visualization of indolamines (IA), a two-step FA vapour treatment is recommended (cf. Fuxe and Jonsson 1967). First treatment takes place at $+80^{\circ}\text{C}$ for 1 h with paraformaldehyde equilibrated in 50% relative humidity, followed by a second treatment (1 h, $+80^{\circ}\text{C}$) with paraformaldehyde equilibrated in 70–90% relative humidity. For details on the humidity equilibration of paraformaldehyde, see Hamberger (1967). The paraffin-embedded specimens are stored in the dark and at a cool temperature (preferably in a freezer) until sectioning.

Sectioning. Sectioning is usually performed at 10–20 μm thickness. The sections are mounted on clear, fluorescence-free slides without the use of water. Cover the sections with a 4:1 mixture of Entellan (Merck, Darmstadt, G.F.R.) and xylene. The slides are left on the hot plate for 1 1/2 – 2 h in order to dissolve the paraffin of the sections completely into the mounting medium. The mounted sections should be kept in the dark and at a cool temperature. They can be stored for many months in a freezer (-20°C) without any significant decline in the quality of the fluorescence picture. Note that the sections should not be frozen until the xylene has evaporated from the mounting medium (which usually occurs within 1–2 days at $+4^{\circ}\text{C}$). Otherwise the xylene will form disturbing bubbles in the polymerized Entellan.

Fluorescence microscopy. This is performed at 405/435 nm illumination (e.g. Schott BG 12 as lamp filter) using a secondary barrier filter with 50% transmission at 470–500 nm. For considerations on specificity tests and microspectrofluorometric analysis of the monoamine fluorophores see Björklund et al. (1972a, 1975), and Corrodi and Jonsson (1967) and Paper VI.

Indoleamine visualization. In rat brain the intraneuronal indoleamine fluorescence is very weak unless the intraneuronal indoleamine content is raised through pharmacological pretreatment. This can be done by administering a MAO-inhibitor in a high dose, 3–5 h before killing (nialamide (Pfizer) at a dose of 300 mg/kg, i.p., or pargyline hydrochloride (Sigma) at a dose of 150 mg/kg, i.p.). The increase in intraneuronal indoleamines is even greater after combined MAO-inhibition and L-tryptophan treatment

according to Aghajanian et al. (1973): chloral hydrate (300 mg/kg, i.p.) followed 10 min later by nialamide (300 mg/kg i.p.) and 5 min later by L-tryptophan (100 mg/kg, i.p.). The rats are killed 1-3 h after the last injection.

II. The ALFA method: young animals (Paper V)

The young animals are perfused by means of a pressurized perfusion system, which is somewhat modified compared to that used for adult animals (described in detail in Paper V).

A. Perfusion procedure. The rat pups and young rats are perfused in two steps (see I:A above).

The pre-perfusion solution consists of a room-tempered Tyrode's buffer with 2 g $\text{MgSO}_4 \times 7 \text{H}_2\text{O}$ and 1 g procain chloride added per liter. The main perfusion (the aluminum perfusion) consists of an ice-cold Tyrode's buffer with 10-20 g $\text{Al}_2(\text{SO}_4)_3$ (the younger the rats the higher the concentration), 0.38 g $\text{Na}_2\text{B}_4\text{O}_7$ and 2% FA added per 100 ml buffer.

The pre-perfusion is introduced with a pressure of $0.3 - 0.5 \text{ bar/cm}^2$ and continued for 45-60 secs. The main perfusion is then carried out for 2-4 min during which the pressure is gradually increased to $1.0 - 2.0 \text{ bar/cm}^2$ (the higher pressures are used for older rats). The perfused volumes of the pre- and main perfusion solutions are at least 20 and 80 ml, respectively. In older animals (over 2 weeks of age) it is recommended to clamp the descending aorta when brains are going to be processed for fluorescence microscopy. Marked swelling often occurs if the descending aorta is clamped in young animals.

The specimens are further processed as described above (see I:B-E above)

F. Pharmacologic pretreatment. To further improve the CA and 5-HT visualization the animals are pretreated with either the MAO inhibitor, pargyline (hydrochloride, Sigma, 150 mg/kg, i.p., 2-3 h) in order to increase endogenous monoamine levels or α -methyl-NA (hydrochloride, Regis, 25-100 mg/kg, s.c., in 0.1% ascorbic acid saline pH 5.0, 1-2 h) was administered 5-15 min after the α -receptor blocker, phentolamine (Regitine^R, CIBA-Geigy, 10-35 mg/kg, i.p.). In young animals (up to 7 days of age) α -methyl-NA can be administered subcutaneously due to immaturity of the blood-brain barrier. Pretreatment with the α -receptor blocker is recommended in order to avoid death due to cardiovascular and pulmonary side effects. The animals are also routinely pretreated with heparin (50-200 units i.p.)

2-10 min prior to perfusion.

III. The Mg-FA-GA method: adult animals (Paper I)

A. Perfusion procedure. The animals are perfused in two steps by means of a syringe (adult animals with a 150 ml syringe). A pre-perfusion step is not necessary, since the perfusate does not cause intravascular precipitation, but gives minor improvements of the fluorescence picture, e.g., improved tissue morphology and a brighter fluorescence intensity of the CA and 5-HT systems. The perfusion pressure should be kept high. Too low a perfusion pressure gives inferior results.

The pre-perfusion solution consists of 150 ml of an ice-cold Tyrode's or KRB buffer (pH 4.5) and the main perfusion (the magnesium perfusion) consists of 150 ml of an ice-cold phosphate buffer with a) 40 g $\text{MgSO}_4 \times 7 \text{H}_2\text{O}$, 2% GA and 0.5% FA added (pH 4.5) or b) 40 g $\text{MgSO}_4 \times 7 \text{H}_2\text{O}$ with 4% FA added (pH 4.5).

The pre-perfusion is not necessary in the Mg-FA-GA method but offers some advantage (Lorén et al. 1977). The perfusions are carried out with a high pressure, and the descending aorta is clamped. A pressurized perfusion system similar to that designed for the ALFA method has been tested in the Mg-FA-GA method (cats and rabbits) and can be recommended.

B-E: Freezing, processing, sectioning and fluorescence microscopy are carried out as described above (I B-E).

F: Pharmacological pretreatment for indolamine visualization is carried out as described above (I F).

IV. The Mg-FA-GA method: young animals (Paper V).

A. Perfusion procedure. The rat fetuses and young rats are perfused in two steps. The pre-perfusion solution consists of a) 20 ml of an ice-cold Tyrode's or KRB buffer pH 4.5 or b) 20 ml of a room-tempered Tyrode's or KRB buffer with MgSO_4 and procain added (see II:A above) The pre-perfusion step is not necessary. The main perfusion solution is identical to that used for adult animals. The young rats are perfused with 20-40 ml of the Mg-FA-GA solution. The specimens are further processed as described above (see I: B-E). Pharmacological pretreatment is performed as described above (see II:F).

Cryostat techniques

I. The ALFA method (Paper III and IV)

The animals are perfused by means of the pressurized system as described above (see also Paper III).

A. Perfusion procedure. The animals are perfused in two steps, first with a pre-perfusion and then with the main perfusion (the aluminum-containing) solution. The pre-perfusion solution consists of a) 150 ml of an ice-cold Tyrode's or KRB buffer (pH 7.2) or b) 150 ml of an ice-cold 2% GA solution (pH 7.0). The main perfusion solution consists of 300-450 ml of an ice-cold Tyrode's buffer with 30 g $\text{Al}_2(\text{SO}_4)_3 \times 18\text{H}_2\text{O}$, 1.14 g $\text{Na}_2\text{B}_4\text{O}_7 \times 10\text{H}_2\text{O}$ and 1-4% FA added per 300 ml.

For studies on the forebrain and brain stem the animals are perfused with 300 ml of the main perfusion solution with the descending aorta clamped. Larger volume (450 ml) is used for spinal cord, PNS, endocrine and visceral organs. 2,5-5% sucrose is added to the respective perfusion solution to improve the morphology of the sections.

B. Freezing and sectioning. Perfused and unperfused tissue are rapidly dissected and glued to or frozen onto a cryostat chuck, which then is inserted in powdered dry-ice. The tissue pieces are then stored in the cryostat at about -25 to -30°C (CNS tissue) or at about -15 to -20°C (PNS, endocrine and visceral organs) to equilibrate to the temperature of the cryostat. Sectioning of CNS tissue is carried out at a temperature of about -25 to -30°C to obtain optimal fluorescence picture. Higher temperatures cause diffusion and decrease of the CAs that are visualized. For the demonstration of CAs and 5-HT in neuronal and non-neuronal storage sites, e.g., endocrine and visceral organs, both perfused and unperfused tissues are used. In this case it is possible to achieve a good fluorescence picture with a higher sectioning temperature of about -15 to -20°C , which gives an improved tissue morphology. The tissue pieces are sectioned at 15 - $20\text{ }\mu\text{m}$, and the sections are then picked up and thawed on room tempered microscope slides.

C. Immersion. Immersion of unperfused tissue is carried out for 30-60 sec in an ice-cold Tyrode's or KRB buffer with 2.5 g $\text{Al}_2(\text{SO}_4)_3 \times 18\text{H}_2\text{O}$ added per 100 ml buffer. The aluminum salt adjusts pH of the solution. The immersion is performed

in a Coplin jar.

D. Drying, FA vapour treatment, mounting of sections. The sections are dried under the warm air stream from a hair-dryer for 5–15 min, and then in vacuo in a desiccator over P_2O_5 for at least 2 h. The sections are finally treated with FA vapour at 50–70% humidity for 1 h at $+80^{\circ}C$, mounted in paraffin oil or Entellan (Merck, Darmstadt, G.F.R.) and taken for fluorescence microscopy.

II. The Mg-FA-GA method (Paper II)

The animals are perfused by means of a 150 ml syringe and with a high pressure.

A. Perfusion procedure. The animals are perfused with 150 ml of an ice-cold phosphate buffer with 40 g $MgSO_4 \times 7 H_2O$, 2% GA and 0.5% FA added (pH 4.5). The perfusion is done within 1/2 – 1 min and the descending aorta clamped during the first 25–50 ml of the perfusion.

B. Freezing and sectioning. The rat brains are rapidly dissected and glued or frozen onto a cryostat chuck inserted in powdered dry ice or immediately placed in the cryostat chamber, set at $-30^{\circ}C$. All tissue pieces are kept in the cryostat for about 30 min. The tissue pieces are sectioned at 15–20 μm thickness and picked up and thawed on room-tempered glass slides.

C. Immersion. A short immersion (30 sec) is carried out in an ice-cold 2% GA solution (pH 7.0).

The sections are further processed as described above (see I:D)

Vibratome techniques

I. The ALFA method (Paper III)

A. Perfusion procedure. The animals are perfused with 150–1000 ml of an ice-cold 4% FA solution (pH 7.0) either by means of a syringe (volumes of 150–300 ml) or by means of a pressurized perfusion system (the larger volumes) at a moderate pressure.

B. Sectioning. The brains are rapidly dissected, glued to the Vibratome holder, and then sectioned at 30–40 μm according to the description given by Hökfelt and Ljungdahl

(1972) and Lindvall and Björklund (1974). The specimens are immersed in an ice-cold, oxygenated Tyrode's or KRB buffer during sectioning. For further aspects on the Vibratome sectioning, see Lindvall and Björklund (1974).

C. Immersion. This is carried out in 150 ml of an ice-cold Tyrode's or KRB buffer with 2.5 g $\text{Al}_2(\text{SO}_4)_3 \times 18 \text{H}_2\text{O}$ added per 150 ml buffer. The initial pH is 7.2-7.4 and added aluminum salt adjusts pH. The sections are kept free-floating below the surface, and the optimal immersion time is 15-30 sec.

D. Drying, FA vapour treatment, mounting of sections. The sections are dried under the warm air stream from a hair dryer for about 15 min, and then over P_2O_5 in a dessicator in vacuo for 2-24 h. They are finally reacted with FA vapour for 1 h at $+80^\circ\text{C}$, using paraformaldehyde equilibrated in air of 50-70% humidity. The sections are cover-slipped with liquid paraffin as mounting medium and analyzed in the fluorescence microscope.

II. The Mg-FA method (Paper II)

A. Perfusion procedure. The animals are perfused with 150-1000 ml of an ice-cold 4% FA solution with 2-5 g $\text{MgSO}_4 \times 7 \text{H}_2\text{O}$ added per 100 ml. The added magnesium salt adjusts pH of the perfusion solution. The perfusion is carried out by means of a syringe or a pressurized perfusion system (see ALFA method above).

B. Sectioning. See ALFA method, I B above.

C. Immersion. The immersion is carried out for about 1-2 min in an ice-cold Tyrode's or KRB buffer with 4.1 g $\text{MgCl}_2 \times 6 \text{H}_2\text{O}$ or 5 g $\text{MgSO}_4 \times 7 \text{H}_2\text{O}$ dissolved per 150 ml buffer. The addition of magnesium salts adjusts pH of the solution. The sections are kept free-floating below the surface during the immersion. The immersion time in MgCl_2 can be prolonged up till 10 min without any changes of the fluorescence picture; L. Kromer, personal communication.

D. Drying, FA vapour treatment, mounting of sections. See I D above.

Whole mounts

I. The ALFA and Mg-FA-GA methods: perfused tissue (Paper IV)

A. Perfusion procedure. The animals are perfused according to the ALFA or Mg-FA-GA method (see freeze-drying, paraffin embedding or cryostat techniques above).

B. Preparation. The tissue samples are dissected and stretched onto microscope slides. The specimens are then further processed as described above; see Vibratome techniques, the ALFA method, I:D.

II. The ALFA method; unperfused tissue (Paper IV)

A. Immersion. The tissue samples are dissected and immersed in an ice-cold aluminum sulphate containing solution ($2.5 \text{ g Al}_2(\text{SO}_4)_3 \times 18 \text{ H}_2\text{O}$ dissolved per 100 ml of a Tyrode's or KRB buffer) for 3-5 min. The tissue samples are then stretched onto microscope slides. The specimens are then further processed as described above; see Vibratome techniques, the ALFA method, I:D.

DISCUSSION

This review has summarized improvements of the fluorescence histochemical methodology based on condensation with FA, alone or together with GA, in combination with high concentrations of metal ions, for sensitive visualization of intraneuronal NA, DA and 5-HT in tissue samples processed via one of the routine procedures for histochemical processing, i.e. freeze-drying and paraffin embedding, cryostat sectioning, Vibratome sectioning or whole-mount preparations.

The first observation made was that high concentrations of magnesium ions, introduced via intravascular perfusion, markedly enhanced the sensitivity for detection of NA, DA and 5-HT in CNS tissue. These findings were used for an improvement of the original Falck-Hillarp method, i.e. the Mg-FA-GA histofluorescence method for freeze-dried, paraffin embedded specimens. Subsequently, the same principles were applied to cryostat and Vibratome sections. The most clear-cut advantage of the Mg-FA-GA histofluorescence method, as well as a sign of its high sensitivity, was its successful application in ontogenetic studies on developing CA systems, which was the field where the need for new, highly sensitive methods has been obvious.

In the search for an explanation for the positive effects exerted by the magnesium ion, the interest was focused upon several different possible factors. The most obvious possibility was that the magnesium ion, which is a strong Lewis acid, acted via acid catalysis. It has previously been shown that the fluorophore formation from monoamines in both the FA and GA reactions are catalysed by acids (for further comments see Björklund et al. 1975). Consequently, weaker and stronger Lewis acids could be supposed to possess lower and higher fluorescence-promoting effects, respectively. This was, in fact, seen in sections from parallelly processed tissue samples. Thus, the aluminum ion, which is a stronger Lewis acid than magnesium and sodium ions, also had a stronger fluorescence promoting effect compared with these ions. On the basis of these findings a highly sensitive fluorescence method - the ALFA method - was developed for freeze-drying and paraffin embedding, cryostat sectioning, Vibratome sectioning and whole-mount preparations. As was the case for the Mg-FA-GA method, the ALFA method proved

to be ideal, and in fact superior to the magnesium method for ontogenetic studies. Within this field the ALFA method thus allows a much more detailed study of central nervous CA:s and 5-HT neurons.

The effects of perfusion with high concentrations of aluminum and magnesium ions are probably manifold. Minor positive effects are obtained by "non-specific" factors, such as tissue dehydration, lowering of pH and introduction of the reagents FA and GA into the tissue. Perfusion with hypertonic solutions causes dehydration of the tissue, thereby probably reducing the risk of diffusion artefacts. Perfusion with acid isotonic solutions gives minor increase in fluorescence intensity, but also an increased diffusion of the intraneuronal CA:s. This diffusion might be explained by the observations that the release of CA:s from adrenal medullary vesicles is increased in parallel with increased acidity of the incubation

(cf. Philippu 1976) Perfusion with ice-cold isotonic solutions

(as well as immersions in ice-cold solutions) also gives some minor improvements of the fluorescence picture. This might be possible to explain by the findings that the CA content of adrenal vesicles remain stable for longer periods at low temperature, 0°C (cf. Philippu 1976). Perfusion with the reagents FA and GA alone or in combination, might help to bind the amines within their storage sites and may also facilitate the FA condensation step by initiating the Pictet-Spengler cyclization step and thereby the fluorophore formation.

The main effects seen in the present procedures are by all probability created by the aluminum and magnesium ions themselves. Magnesium ions are known to inhibit release of transmitter from both central and peripheral adrenergic nerves (Boullin 1967, Kirpekar and Misu 1967, Farnebo 1971). Together with ATP, magnesium ions are known to enhance the uptake of CA:s into storage vesicles of cells from the adrenal medulla (Carlsson et al. 1963, Philippu 1976). These effects are seen at much lower concentrations than those used in the Mg-FA-GA method. However, it is conceivable that one effect of the magnesium ions is to "lock in" the CA:s and to prevent their spontaneous release from their storage sites. This is the probable explanation for the extreme sharpness and lack of diffusion within the fluorescence picture in combination, perhaps with the dehydrating effect of the hypertonic perfusion. This so called "locking-in" effect could also be exerted by the aluminum ions since the fluorescence picture - extreme sharpness and lack of diffusion in the

fluorophore localization - obtained with the ALFA method has the same features. It is not known, however, if aluminum ions have any inhibitory effects on the release of transmitter. The "locking-in" effect may result in a very precise localization of the amines and later of the fluorophores in tissue and preservation of maximum amount of amines and fluorophores during histochemical processing.

Acid catalysis is of importance both for the FA and GA reactions to increase the fluorescence yield. External acid catalysis is performed with HCl-vapour in the FA reaction (Björklund and Stenevi 1970, Björklund et al. 1971, 1972a, 1975). Internal acid catalysis is performed in the GA reaction by the GA molecule itself (Lindvall et al. 1974). This is in all likelihood also the case in the Mg-FA-GA and the ALFA methods. Since the magnesium and aluminum ions are strong Lewis acids they probably participate in the FA reaction in a way similar to the action of the GA molecule; i.e. acting via internal acid catalysis. This is seen also in the reaction with melatonin in the GA and ALFA methods although the GA and the aluminum ions do not react identically. Two important direct effects of the aluminum and magnesium ions can be exerted in the ALFA and Mg-FA-GA methods: a) a direct action on the fluorophore reactions and b) a direct action on the fluorophores formed. As Lewis acids, these ions can participate as internal catalysts in the formation of tetrahydroderivatives via the Pictet-Spengler cyclization step. They can also act as acid catalysts in the second reaction step, where the fluorophores are formed. In this step amino acids and probably also Lewis acids act as acid catalysts in the reaction between a second FA molecule and the tetrahydroderivatives to form the strongly fluorescent 2-methyl-dihydroderivatives (2-methyl-dihydroisoquinolines and 2-methyl-dihydro- β -carboline). The 2-methyl-dihydroderivatives have a higher fluorescence yield, about 2-3 fold (Björklund et al. 1973a). Thus, there is a possibility for these metal ions to promote the formation of strongly fluorescent 2-methyl-dihydro-derivatives at the expense of dihydroderivatives formed via autoxidation.

Finally, in the model experiments the fluorescence promoting effect, caused by magnesium and aluminum ions, was shown primarily to be due to a direct interaction with the fluorophores formed in the FA reaction, leading to an amplification of their fluorescence i.e. an increased quantum efficiency. This could perhaps be explained by an advantageous complex binding between magnesium or aluminum ions and several of the formed fluorescent molecules, which thereby exhibit stronger fluore-

science. However, the entire action of magnesium and aluminum ions within the developed fluorescence methods cannot be explained solely on basis of the results obtained from the model experiments. The fluorescence yield in model droplets was increased twice in DA and reduced in NA containing droplets. In central nervous tissue processed according to the ALFA method the increase was 3-4 times for DA and NA (unpublished observation). Thus, certain mechanisms probably act in the perfused tissue that cannot be reproduced in a protein matrix.

The fact that a fluorescence promoting effect, i.e. a high quality of the fluorescence picture, is seen at low equimolar concentrations with both AlCl_3 , $\text{Al}_2(\text{SO}_4)_3$, MgCl_2 and MgSO_4 but only with $\text{Al}_2(\text{SO}_4)_3$ and MgSO_4 at high concentrations suggests that the chloride ion have some negative effects at this level. The fluorescence picture showed disturbing diffusion of CA:s and 5-HT although the fluorescence intensity was rather strong. It has been reported that chloride ions enhance the release of CA:s from their storage vesicles in the adrenal medulla, even in the presence of magnesium ions, by increased permeability of the vesicle membranes (Philippu 1976). The sulphate ions may either be neutral in this respect or they might even exert a membrane stabilizing effect, thereby participating in the above-mentioned "locking-in" effect. As the sulphate ion is a weak base it does not participate in the acid-catalyzed FA reaction. It is not known if the hydrogen sulphate (HSO_4^-) ion, which is a strong acid exists in the perfusate and thereby participate in the aluminum or the magnesium ion acid catalyzed FA reactions. Thus, the role of the sulphate ions, if any, remains obscure.

The aluminum- and magnesium-catalyzed methods, i.e. the ALFA and the Mg-FA-GA methods, have several advantages over other alternative methods. Together with the GA methods (Lindvall and Björklund 1974, Furness and Costa 1975) they represent the most sensitive fluorescence histochemical aldehyde methods now available. They are undoubtedly the most sensitive procedures developed for freeze-drying and paraffin embedding as well as for cryostat sections. Together with the GA method they are the most sensitive methods for demonstration of central CA neurons when applied to Vibratome sections. The Mg-FA method is the least sensitive, which is seen in its insufficiency to demonstrate systems with low amine content, such as the DA-containing incerto-hypothalamic and anterior cingulate cortex systems (Lindvall and Björklund 1978). Moreover, the ALFA, Mg-FA-GA and Mg-FA

methods, together with the GA methods (Lindvall and Björklund 1974, Furness and Costa 1975) and the Faglu method (Furness et al. 1973b), represent the most sensitive methods used for demonstration of CAs and 5-HT in whole-mount preparations.

In addition to their high sensitivity, the ALFA and Mg-FA-GA methods offer advantages over the GA-Vibratome method when the freeze-drying and paraffin embedding technique is used. First, it is possible to obtain serial sectioning of large tissue blocks. This allows serial sectioning through those areas, which require the utmost sensitive fluorescence methods for their detailed neuroanatomical description, e.g. for mapping and lesion studies. This is most obvious for such systems as the tubero-infundibular system, the incerto-hypothalamic system, and the anterior cingulate and prefrontal cortex systems. Naturally, such sensitive methods should also be used for neuroanatomical studies of the other CA systems in the brain. Second, there are no restrictions for obtaining complete section series of consistent quality, which is a problem in the Vibratome method due to the composition of certain tissues, e.g. endocrine and visceral organs. Thus, the DA-containing nerve fibers of the intermediate and posterior lobes of the pituitary, dissected from pargyline pretreated rats and processed according to the most optimal ALFA method, were demonstrated with at least the same high sensitivity as that previously seen in rats pretreated with α -methyl-NA and processed according to a modified Falck-Hillarp method (Björklund and Lörén unpublished observations; for a detailed information of this system, see Björklund et al. 1974). Furthermore, the tryptophan-containing peptide in the ACTH cells of the anterior lobe and the α -MSH cells of the intermediate lobe, which previously has been visualized with modified FA reactions (Björklund and Falck 1969, Håkanson et al. 1972, 1974, 1975) and with the GA method (Björklund et al. 1973b), was clearly demonstrated with the ALFA method. Since the introduction of the original Falck-Hillarp method the ALFA method represents the first major improvement for the demonstration of CAs and 5-HT in neuronal and non-neuronal storage sites of visceral and endocrine organs (paper IV). Certain adrenergic nerves of the ovary, the pancreas (see also Sundler et al. 1980, Ahrén et al. 1981), the thyroid (see also Sundler et al. 1980) and the gastrointestinal tract, as well as SIF cells and 5-HT-storing enterochromaffin cells, were demonstrated with a precision and richness of details hitherto not seen. The adrenergic nerves of the rat ovary were visualized with the same high sensitivity and

the same extent as later been revealed with the GA-Vibratome method (Stefenson et al. 1981). The ALFA method is also excellent for demonstration of APUD cells after L-dopa administration and for visualization of aldehyde induced fluorescence as well as immunohistochemistry in the same section (Sundler et al. 1980).

One further major advantage of the ALFA and Mg-FA-GA methods, compared to other aldehyde histofluorescence methods, is that they are the only techniques that allow a highly sensitive detection of CA:s and 5-HT in the developing brain. A detailed and quantitative analysis of the developmental growth of central CA systems have previously been hampered because of shortcomings of the techniques used (low sensitivity due to the low intraneuronal amine content, diffusion, and difficulties in obtaining an even and reproducible fluorescence picture). These limitations are overcome in the ALFA and Mg-FA-GA methods. The soft consistency, due to high water content, of the immature brain makes sectioning difficult. Thus, sufficiently thin and even sections are difficult, though possible to obtain within the Vibratome techniques. The methods of choice for such tissue are the ones based on the freeze-drying and and paraffin embedding protocol.

When comparing the ALFA and Mg-FA-GA methods with the immunohistochemical techniques utilizing antibodies raised against the different CA-synthesizing enzymes (tyrosine hydroxylase, dopa decarboxylase, dopamine- β -hydroxylase and phenylethanolamine-N-methyltransferase) it appears that the immunohistochemical techniques can have a sensitivity for the visualization of CA neuron systems comparable to that of the metal ion-catalyzed aldehyde methods (cf. Hökfelt et al. 1975, 1976, 1977, Swanson and Hartman 1975). Most studies on developing CA systems in the immature brain have nevertheless been carried out with aldehyde fluorescence histochemical methods. This may be due to the fact that the content of transmitter-related enzyme in immature central CA neurons is as low as that of the transmitter (Coyle and Molliver 1977, Schmidt and Bhatnagar 1979). Thus, the existing potential for increasing the neuronal content of transmitter, either endogenously or exogenously, would seem to give the aldehyde fluorescence histochemical methods an advantage in sensitivity.

The Mg-FA-GA method has been successfully used in several studies for the demonstration of CA:s and 5-HT in the developing rat brain (Lorén et al. 1976, Lidov et al. 1978, Levitt and Moore 1978, 1979, Loy and Moore 1979). The ALFA method



is more sensitive than the Mg-FA-GA method. In particular, it offers an improvement in the visualization of CAs in several areas of the brain, e.g., in cerebellum, cerebral cortex, hippocampus and the spinal cord. The hyperinnervation pattern, as revealed in the prefrontal cortex, the cerebellum and the anterior septum and the hippocampal rudiment, represent new findings and are at present subjected to further investigation (manuscripts in preparation). Moreover, such areas as cerebellum, cerebral cortex and hippocampus exhibited in the newborn rat a much denser innervation pattern than previously described. By contrast, the terminal areas of the incerto-hypothalamic and the anterior cingulate DA systems are only weakly developed at postnatal day 7-8. The nerve cell bodies of the incerto-hypothalamic system, but no terminals, are seen at birth.

The Mg-FA-GA and ALFA methods allow a somewhat improved visualization of 5-HT-containing nerve cell bodies, as well as of their processes, but only marginal improvements for the demonstration of terminals, when compared with the standard Falck-Hillarp method. The main advantages are brighter fluorescence intensity and better precision in the visualization of 5-HT-containing neurons. Perfusion with metal salts ($\text{Al}_2(\text{SO}_4)_3$ or MgSO_4), in combination with the reagents FA or GA, do not help to stabilize the 5-HT fluorophores. Thus, the rapid fading of 5-HT is also seen in specimens treated according to these new methods. The method of choice for the visualization of 5-HT is, therefore, the immunohistochemical technique based on antibodies raised against serotonin (Steinbusch et al. 1978). This method has made it possible to obtain a more detailed, and in several respects new and more complete anatomical description of the 5-HT systems in the rat brain (Steinbusch 1981).

A special, interesting feature of the ALFA method, which distinguishes it from the magnesium-catalyzed procedures, is the efficient fluorophore formation from tertiary indolamines (such as bufotenin) and N-acetylated indolamines (such as melatonin). Although the model experiments show that the ALFA method should be sensitive for visualization of such amines in tissue, the observations made so far in various regions of the rat CNS have failed to demonstrate any fluorescent structures beside the CA-containing and the 5-HT-containing ones. The possibilities to use the ALFA method for histochemical visualization of N-acetylated and tertiary indolamines are thus still unclear.



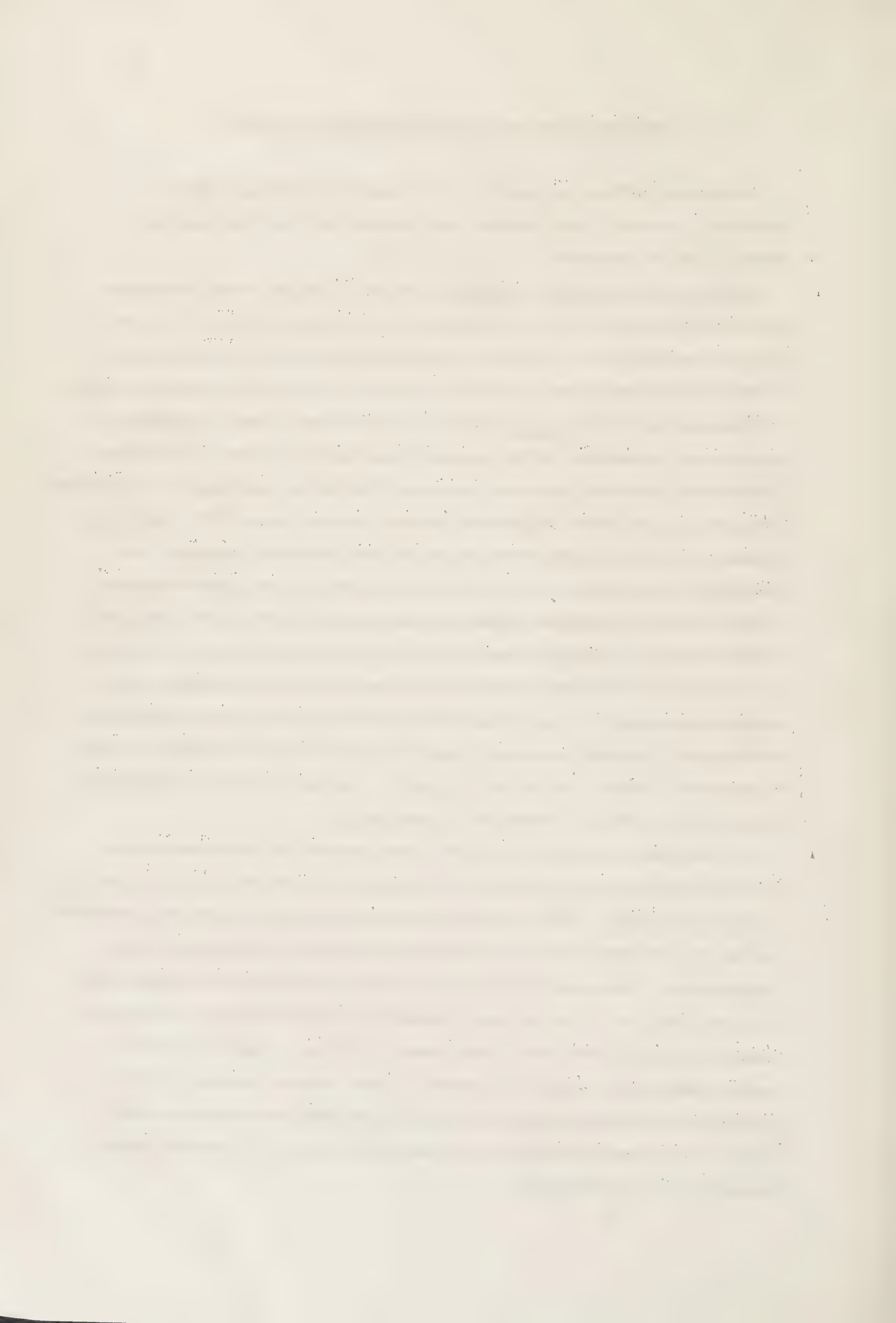
Comments on the use of different alternative methods

A number of different and sensitive histofluorescence techniques are now available. They are in many respects complimentary and have advantages and, hence, fields of application.

Freeze-drying and paraffin embedding. This technique has several advantages over other procedures, such as the cryostat and Vibratome techniques. First, the structural preservation of CA and 5-HT-containing cell bodies is better than seen in the Vibratome techniques. Secondly, all kinds of tissue can be processed according to this technique. Third, en bloc reaction is the only way to obtain consistent and reproducible fluorescence yields throughout large series of sections and throughout large series of parallelly processed specimens. Fourth, many specimens can be processed simultaneously and several regions can be taken from each brain. Fifth, the en bloc processing allows very convenient storage and transportation of specimens after freezing of the specimens. In addition, the specimens can once paraffin embedded again be stored for prolonged periods in a deep-freeze. Sixth, paraffin sectioning allows complete, uninterrupted serial sectioning of large tissue blocks. Furthermore, ALFA treated material can be used with excellent results for simultaneous or consecutive demonstration by use of immunohistochemistry, various histo-pathochemical stainings and fluorescent retrograde tracers in combination with an aldehyde induced fluorescence. Seventh, this is the only method for highly sensitive demonstration of central CA:s and 5-HT systems during ontogenesis.

The most sensitive methods to be used in the freeze-drying technique are the aluminum-catalyzed ALFA method and the magnesium-catalyzed Mg-FA-GA method.

Cryostat technique. This technique has several advantages but also some drawbacks, such as variable reproducibility, uneven section morphology, and low sensitivity. Advantages with the cryostat technique are, first, that the cryostat instrument allows serial sectioning and is the instrument accessible in most laboratories. Second, it is useful when maximal sensitivity is not needed. In the best cryostat methods the optimal sensitivity for central CA systems is higher than that obtained with the standard Falck-Hillarp method for freeze-dried and paraffin embedded material. Third, it allows rapid processing and access to microscopy of the sections within a couple of hours after dissection.



In our hands, the most sensitive methods in this technique have been the aluminum and magnesium-catalyzed methods, i.e. the ALFA method and the Mg-FA-GA method.

Vibratome technique. This technique is together with the freeze-drying and paraffin embedding technique the most sensitive technique for demonstration of CA:s in the CNS. The drawbacks of this technique are: it does not produce large series of sections and sectioning is often difficult (most prominent in the GA-Vibratome method). It does not allow sectioning of all kinds of tissue. The Vibratome technique has, however, several advantages. First, it has a very high sensitivity for demonstration of central CA systems. Second, it allows a high preservation of the CA-containing structures and of the section morphology. Third, it does not need all the equipment and chemicals necessary in the freeze-drying technique. Fourth, it allows rapid processing and access to microscopy of the sections within a couple of hours after dissection.

The most sensitive methods to be used in this technique is a) the GA-Vibratome method (Lindvall and Björklund 1974), b) the aluminum-catalyzed ALFA method and c) the magnesium-catalyzed Mg-FA method. The GA-Vibratome and ALFA method are the most sensitive ones and seem to be interchangeable for neuroanatomical studies. The ALFA method is technically the easiest to handle, while the GA-Vibratome method allows differentiation between NA and DA preterminal axons and terminals due to morphological differences of these structures.

Whole-mount preparations. This technique, which is suitable for tissues that can be stretched as thin sheets or for tissue that can be divided into separate layers, have several advantages: first, it is a highly sensitive technique for demonstration of CA:s and 5-HT in peripheral organs. Second, it allows rapid processing and access to microscopy of the sections within a couple of hours after dissection. Third, it gives a complimentary neuroanatomical picture when used in combination with other techniques. Fourth, it does not require any special equipment, such as freeze-dryer, cryostat, or Vibratome instrument.

The most sensitive methods to be used are a) the GA-methods based on perfused and/or immersed tissue (Lindvall and Björklund 1974, Furness and Costa 1975), b) the aluminum-catalyzed ALFA method for perfused or immersed tissue, c) the magnesium-catalyzed Mg-FA-GA method for perfused tissue, and d) the Faglu method based on immersed tissue (Furness et al. 1977b).



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